University of Alberta

Defective Wound Healing

by

Barbara Sharon Bauer

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ABBREVIATIONS

%	percent
α	alpha
β	beta
γ	gamma
μ	micro
°C	degree Celsius
18S	sedimentation coefficient (Svedberg units) of an rRNA component
AdipoR1/AdipoR2	adiponectin receptors 1 and 2
AdTGF-B1	adenovirus containing latent transforming growth factor beta-1
AdTGF-B1$^{223/225}$	adenovirus containing active transforming growth factor beta-1
ATP	andenosine 5'-triphosphate
bFGF	basic fibroblast growth factor
BMI	body mass index
BMP	bone morphogenic protein
BSA	bovine serum albumin
Cal	calorie
CAR	coxsackievirus and adenovirus receptor
CD4	T helper cell subpopulation
CML	chronic myelogenous leukemia
cp	corpulent
CS	chondroitin sulfate
Cys	cysteine

d	day
db/db mice	strain of mouse with defective leptin receptor
dFBS	dialyzed fetal bovine serum
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DS	dermatan sulfate
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbant assay
fa/fa	fatty, refers to Zucker fatty rat, a rat strain with mutated leptin receptors
FBS	fetal bovine serum
FFA	free fatty acid
fig	figure
FPCL	fibroblast populated collagen gel
g	gram
GAG	glycosaminoglycan
GITC	guanidinium thiocyanate
Gln	glutamine
GLUT	glucose transporter
h	hour
H&E	hematoxalin and eosin stain for histology
HA	hyaluronic acid

HCl	hydrochloric acid
HDL	high density lipoprotein
HSc	hypertrophic scarring
IFN	interferon
IGF	insulin-like growth factor
IGFBP	insulin-liike growth factor binding protein
IgG	immunoglobulin gamma
IL	interleukin
IRS	insulin receptor substrate
JAK	Janus kinase
JCR:LA-cp/cp	strain of rat with defective leptin receptor
k	kilo
KSFM	keratinocyte serum-free medium
L	litre
LAP	latency associated protein
LDL	low density lipoprotein
LTBP	latent transforming growth factor binding protein
LTGF-β	latent transforming growth factor-beta
m	meter
m	milli
M	molar
m/z	molecular weight/ion ratio
M6P	mannose-6-phosphate

MAP	mitogen activated protein
MCP-1	macrophage chemoattractant protein-1
MEDICA16	β,β'-tetramethylhexadecanedioic acid
MIF	macrophage migration inhibitory factor
min	minute
MIP-2	macrophage inflammatory protein-2
MLEC	mink lung epithelial cells
MMP	matrix metalloproteinase
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
n	nano
N	normal
NaOH	sodium hydroxide
NFκB	nuclear transcription factor-κB
NO	nitric oxide
O_2	oxygen
ob/ob mice	murine strain with defective leptin protein
OB-R	leptin receptor gene
OBRb	leptin receptor spice variant b
p	pico
P	probability
PAI/L assay	plasminogen activator inhibitor / luciferase assay
PAI-I	plasminogen activator inhibitor-I

PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with tween 20
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PG	proteoglycan
PGE_2	prostaglandin
PI3-K	phosphatidylinositol-3 kinase
pM9A	pro-MMP-9 activator
PPAR-γ	peroxisome proliferator-activator receptor γ
Pro	proline
RI, RII	transforming growth factor-β receptor type I, type II
RIA	radioimmunoassay
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
S15261	compound for treatment of type 2 diabetes, L-isomer of 2-({2-methoxy-2-[3-(trifluoromethyl)phenyl]ethyl}amino)ethyl-4-(2-{[2-(9*H*-9-fluorylenyl)acetyl]amino}ethyl)benzene
SD	standard deviation
SDS	sodiumdodecyl sulfate
Ser	serine
SHP-1	Src homology phosphatase-1
SSC	sodium chloride/sodium citrate
αSMA	alpha smooth muscle actin

STAT	signal transducer and activator of transcription
TAC	triamcinolone acetonide
$TCID_{50}$	tissue culture infectious dose 50
TGF-β	transforming growth factor-beta
Th1/Th2	T cell helper subsets 1 and 2
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor alpha
t-PA	tissue type plasminogen activator
Tyr	tyrosine
u-PA	urokinase type plasminogen activator
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
vs.	versus

CHAPTER 1

Fibroproliferative Disorders

A version of this chapter has been published in: Bauer BS, Tredget EE, Scott PG, Ghahary A. Molecular and cellular biology of dermal fibroproliferative disorders. In: Garg HG, Longaker MT, eds. Scarless Wound Healing. New York: Marcel Dekker, Inc, 2000:173-211.

I. INTRODUCTION

A. Clinical Features

Hypertrophic scarring (HSc) and keloids are characterized as dermal fibroproliferative disorders, which also include liver cirrhosis and fibrosis, pulmonary fibrosis, atherosclerotic disease of vasculature and multi-organ/tissue systemic diseases such as progressive systemic sclerosis (scleroderma) and rheumatoid arthritis. Keloids can form following either major injury or minor trauma to the skin such as ear piercings or acne; whereas, HSc scars usually form following injury to the deep dermis (1,2). The highest incidence of HSc occurs on body surfaces that are subject to high tension such as the anterior chest, shoulders, flexor surfaces of the extremities and the anterior neck (3). HSc and keloids are characterized by excess extracellular matrix deposition in the dermis and subcutaneous tissues and unlike normal wound repair which results in a fine-line scar, keloids and HSc form hard, elevated, red and sometimes itchy scars which may cause pain, disfigurement and contractures (fig. 1-1).

It is important to distinguish HSc and keloids as the treatments vary. The key differences are that HSc remain within the confines of the wound margin and eventually undergo partial resolution spontaneously; whereas, keloids are capable of invading surrounding tissue and usually require medical intervention as they rarely regress spontaneously. There is believed to be a genetic (4,5) and racial predisposition for the development of keloids in darker skinned races as 15-20% of Blacks, Hispanics and Orientals are afflicted with the disorder. Children entering puberty and pregnant women

are reportedly more susceptible to increases in keloid size, which may be associated with changes in hormone levels.

B. Cellular Biology of Wound Healing

Immediately following injury, a number of sequenced events occur leading to the release of growth factors and cytokines which mediate subsequent inflammation, cell proliferation, extracellular matrix deposition, contraction and remodeling (fig. 1-2). HSc and keloids are the result of aberrations in the normal progression of healing leading to excessive extracellular matrix deposition and often the formation of contractures. Contractures are the pathological shortening of scar tissue which results in cosmetic and functional deformity, as opposed to wound contraction in normal healing which acts to reduce the wound surface area.

Wound healing involves a complex cascade of events in which alterations in key factors can have a great impact on the outcome. After wounding, platelets degranulate and the coagulation and complement pathways are activated to initiate the formation of a fibrin clot that acts as a mesh for the binding of inflammatory cells, fibroblasts and growth factors (6). Platelets release many growth factors which function as chemotactic agents for neutrophils, macrophages, epithelial cells, mast cells, endothelial cells and fibroblasts. These include: epidermal growth factor (EGF) (7), insulin-like growth factor-1 (IGF-1) (8), platelet-derived growth factor (PDGF) (9,10), and transforming growth factor-β (TGF-β) (11). Within 24 hours, neutrophils infiltrate the wound and phagocytose bacteria. Macrophages then infiltrate the wound to clear damaged host cells and debris

and release chemotactic factors for fibroblasts, such as PDGF (12,13) and TGF-β1 (14). PDGF released from platelets and macrophages leads to proliferation and migration of fibroblasts. This process is required for the formation for granulation tissue, which develops from the tissue in the damaged area and consists mainly of small blood vessels, inflammatory cells, fibroblasts, myofibroblasts and ECM proteins (15). Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) promote angiogenesis by stimulating endothelial cell proliferation leading to the formation of capillary tubes. Basic FGF is released by damaged endothelial cells and macrophages (16) and stimulates endothelial cells to release plasminogen activator and procollagenase (17). Plasminogen activator converts plasminogen to plasmin and procollagenase to collagenase, both of which function to digest the basement membrane. Heparin released from mast cells also acts as a chemoattractant for endothelial cells, which are able to migrate to the wound due to the disruption of the basement membrane.

The transformation of granulation tissue into a mature scar requires a balance between matrix biosynthesis and degradation. The degradation of extracellular matrix (ECM) occurs through the action of collagenases, proteoglycanases, and other proteases released from mast cells, macrophages, endothelial cells and fibroblasts. ECM biosythesis is mainly the function of dermal fibroblasts which produce collagens, fibronectin, proteoglycans and other components. A disruption of this balance by either an excess in ECM synthesis or a deficiency in degradation or remodeling may result in the formation of keloids or HSc (18,19) (fig. 1-3).

The development of HSc appears to occur following a prolonged period of inflammation. Prolongation of the inflammatory stage of wound healing due to infection or an extremely large or deep wound leads to an exaggerated response by inflammatory cells and a subsequent excess of cytokine release such as TGF-β and IGF-1, which are fibrogenic cytokines. It has been shown that wounds that do not heal within 3 weeks have a higher probability of HSc formation (20). Keloid formation appears to have a strong genetic predisposition. Oluwasanmi et al. (21) found increased plasma cells and gammaglobulin deposition in Africans and hypothesized that the activity of the immune system may regulate the production of connective tissue during the repair process. Cohen et al. (22) studied keloids and demonstrated an increase in tissue IgG, which indicates that there is an immunological aspect to keloid formation. However, it was not determined what the IgG was directed towards. In these keloid patients, there was no difference in serum IgG or complement levels compared to normal subjects. Cohen and colleagues also reported that there was no correlation between HLA phenotype and keloid formation.

II. CELLULAR FACTORS

A. Fibroblasts and Myofibroblasts

Fibroblasts are the most common cell type found in HSc (23,24). During wound healing, some fibroblasts undergo morphological and biochemical changes such that they have phenotypic characteristics of both fibroblasts and smooth muscle cells. When treated with smooth muscle stimulants, strips of granulation tissue containing these

differentiated cells contracted (25). These cells were subsequently called myofibroblasts to reflect contractile activity of this cell type (reviewed in 26). Gabbiani et al. (27) described the morphological changes that occurred in the fibroblast following wounding. These changes include the formation of stress fibers or microfilaments, nuclear indentations which are often found in contractile cells (28-30), and peripheral attachment sites. Darby et al. (31) demonstrated the gradual evolution of fibroblasts into myofibroblasts using α-smooth muscle actin as a marker. Alpha-smooth muscle actin (α-SMA), an actin isoform found in contractile vascular smooth muscle, is expressed by almost all myofibroblast populations *in vivo* (32).

There has been some debate over the type of cellular motility involved in wound contraction. It has been proposed that myofibroblasts are responsible for wound contraction (33-36) due to the presence of stress fibers rich in actin. Myofibroblasts are interconnected via gap junctions and adhere to the ECM via the fibronexus (37). It was suggested that in granular tissue, many myofibroblasts act as a contractile unit and pull on the connective tissue. Alternatively, single fibroblasts may be capable of reorganizing connective tissue during cell locomotion via tractional forces rather than contractile forces (38,39). It has been proposed that cellular filopodia elongate and retract using tractional forces, a movement resembling that of a tank tread. Attachment sites on fibroblasts have been reported by Izzard and Lochner (40) using interference reflection microscopy. They observed dark streaks where the ventral surface of fibroblasts came in close contact with a glass surface, and they called these streaks focal contacts. Using time-lapsed cinematography, it was shown that the focal contacts remained stationary and

as the cell moved forward, new focal contacts form at the leading edge and pre-existing contacts occupied increasingly posterior positions. These focal contacts have since been equated with adhesions (41). The tractional strength of the fibroblast exceeded that required for locomotion of the cell leading Harris and coworkers to suggest that this process is also involved in rearranging the collagen network from a random one into straight bundles (39). These workers also noted that stress fibers were not required for this process to occur. Herman et al. (42) reported that cellular motility is not usually associated with stress fibers containing actin and myosin but instead, motility occurred when the proteins were diffused and presumably disassembled. Herman and coworkers suggested that these stress fibers anchor the cell to the substrate, as many stress fibers terminate on attachment plaques (40,43) and contraction only occurs after the attachment plaque is released (44,45). Interestingly, when wound contraction is strongest, few stress fibers are expressed. Contraction of fibroblast-populated collagen lattices (FPCL) showed that fibroblasts were most contractile at 48 hours when they had few stress fibers whereas at 96 hours when contraction was minimal, stress fibers were strongly expressed (46). Doillon et al. (47) used a rat model to demonstrate that actin-rich fibroblasts are not directly involved in wound contraction since they are maximally expressed at 15 days after wounding, which corresponds to stability. Alignment of actin filaments in the fibroblasts with new collagen bundles was observed which indicated strong adhesions between the fibroblasts and the ECM thereby facilitating collagen rearrangement. Darby et al., (31) reported that in a rat wound model, stress fibers or microfilaments containing α-SMA appeared on day six while wound contraction was linear beginning on day four. This study also found that the expression of α-SMA was maximal at day 15, at which

point it gradually decreased and was absent by day 30. From day 20-25, apoptotic bodies were noted. Almost certainly fibroblasts have contractile properties and are involved in wound contraction. However, it has been suggested that α-SMA expression corresponds to the end of the migration phase of the myofibroblasts which are then terminally differentiated (48).

Darby et al. (31) and Desmoulière et al. (48) have shown that the number of myofibroblasts undergoing apoptosis increases as the wound closes, resulting in a decrease in cellularity as healing progresses. Basic fibroblast growth factor (bFGF) has been shown to accelerate healing (16) and induce apoptosis in cultured chick embryonic neural retina cells (49), and in oligodendrocytes (50). Basic FGF released from platelets is mitogenic towards fibroblasts, decreases the ability of fibroblasts to contract collagen fibers (51), and induces apoptosis of myofibroblasts. Fibroblasts from rat palatal mucosa were transformed into myofibroblasts with TGF-β treatment *in vitro* in growth-arrested conditions (low serum) (52), and showed a higher level of apoptosis following bFGF treatment. It has been proposed that when granulation tissue cells are not removed, there remains a high degree of cellularity and a subsequent increase in HSc and keloid formation (53). Fibroblasts from hypertrophic scars have shown higher basal levels of contraction in fibrin matrix gels than fibroblasts from keloids and normal dermis which may be due in part to the autocrine effect of TGF-β1 (54) or an increased sensitivity of the fibroblasts to TGF-β1. The extracellular matrix has also been implicated in the phenotypic changes of fibroblasts into myofibroblasts (55). Mechanical tension in the absence of wounding has been shown to induce the myofibroblast phenotype, while in

wounding alone few myofibroblasts were present. In a wound environment, this tension may be provided by the shear forces caused by fibroblast migration (38).

B. Mast Cells

Mast cells are reported to be 4-fold higher in HSc than normal skin and 1.5 times higher in mature scar (56). Mast cells appear as collagen synthesis begins in granulation tissue (57). Histamine release from these cells may contribute to the formation of HSc through vasodilation, which increases the leakage of plasma proteins into the region (58). Mast cells may also contribute to fibrosis by releasing chymase, which has been shown to release the fibrogenic growth factor transforming growth factor-β1, in its inactive or latent form, from the extracellular matrix (59). Smith et al. (60) proposed that histamine and heparin from mast cells may lead to the development of keloids and HSc by increasing the rate of collagen production (61-63). They reported a statistically significant increase in these fibrotic conditions in those individuals suffering from atopic allergies.

III. EXTRACELLULAR MATRIX ABNORMALITIES IN DERMAL FIBROPROLIFERATIVE DISORDERS

A. Collagen

The major classes of extracellular matrix components are collagens, elastic fibers, non-collagenous glycoproteins such as fibronectin, and various proteoglycans. Collagen fibrils and fibers provide tensile strength to connective tissues and an increase in collagen content is usually considered the hallmark of fibrosis. It is worth noting however, that in

hypertrophic scar the proportion of collagen on a dry-weight basis is about 30% lower than in normal dermis or mature scar (64). This is because of the larger increases in other extracellular matrix components such as fibronectin and the proteoglycans (see below). Nevertheless the absolute amount of collagen (expressed per unit surface area) is elevated because of the grossly thickened dermis. In some regions of post-burn hypertrophic scars much of the collagen is organized not (as in normal dermis) into fibers and fiber-bundles running parallel to the tissue surface, but into thin fibrils which can be seen in the electron microscope to be irregular in outline and widely separated by interfibrillar matrix (24). In the light microscope these areas of the scar appear rather fine-textured, with the fibroblasts arranged in "whorls" or "nodules".

The most abundant form of collagen in the skin is type I, with smaller amounts (10-15% in adults) of type III, a collagen that is characteristically a higher proportion of the total in foetal tissues and in early wounds. High and variable levels of type III collagen have been reported in hypertrophic scars (65,66) while both higher (67) and lower (68) proportions of type III collagen have been reported in keloids. Since type III collagen appears very early after dermal injury (within two days) (69) its persistence in hypertrophic scars may reflect their biological immaturity. Collagen fibrils of the dermis are probably mostly mixtures of types I and III collagens (70) and fibrils precipitated in vitro from soluble types I and III collagens are thinner than those made from type I collagen alone (71). Consequently it may be suggested that the higher proportion of type III collagen in hypertrophic scars could account for the thinner fibrils.

Type V is another fibril-forming collagen known to be present in small amounts in skin (72) but which may account for up to 10% of the collagen in hypertrophic scars (73). Immunohistochemistry has been used to localize type V collagen to basement membranes or to the immediately subjacent connective tissue (74,75), so that its elevation in hypertrophic scars may reflect the increased vascularity. However, it is also a component (along with types I and III collagens) of heterotypic fibrils in many tissues including skin and, like type III collagen, it reduces the diameters of copolymeric fibrils formed in vitro (76,77). Its location within the hypertrophic scars has apparently not been defined.

Expression of mRNA for type VI collagen is elevated in keloids compared to normal dermis (78). The type VI collagen monomer consists of a short triple-helical domain flanked by two large globular domains. It assembles into dimers and tetramers which link end-to-end to form thin beaded filaments (79). These filaments are organized into a meshwork oriented approximately perpendicular to the major fibrils in dermis and other connective tissues and are especially prominent in neurofibromatous lesions in the skin (80). It may be speculated that type VI collagen comprises the interfibrillar filaments which are prominent in hypertrophic scars (81).

Collagen fibers in keloids are thicker and are more abundant than are found in HSc and tend to form acellular nodules in the deep dermis. HSc also form nodules or whorls of collagen with fewer distinct collagen fibers and fiber bundles; however, they usually contain islands of α-SMA staining myofibroblasts (82). Another characteristic of

HSc is that there is a lack of epithelial ridges and increased thickness of dermis and epidermis (24). The orientation of the wound may be important in the formation of HSc. Skin tension and collagen organization are directional and wounds which are oriented in the relaxed skin tension lines are protected from stress by collagen fibers and thus form normal mature scars (83).

B. Glycosaminoglycans and Proteoglycans

Proteoglycans (PG) are complex macromolecules in which specific glycosaminoglycan (GAG) chains are attached to a protein core (fig. 1-4). Glycosaminoglycans contain sulfate and carboxylate residues and are therefore highly polyanionic. The predominant GAGs in normal skin are dermatan sulfate (DS) and hyaluronic acid (HA), with smaller amounts of chondroitin sulfate (CS) (84). Chondroitin-4-sulfate is present in very small amounts in normal skin in contrast to HSc in which it is readily demonstrated, especially in the nodules (85).

Proteoglycans impart resilience to connective tissue through their water-holding capacity. In normal skin, decorin is the major PG present (86,87). Decorin consists of a core protein with a single DS chain (88) and 3 N-linked oligosaccharides (89,90). In normal wound healing, decorin expression occurs early and at high levels (91). Decorin is believed to aid in the organization of collagen fibrils into fibers and fiber bundles; delay in decorin expression may result in disorganization of collagen as seen in HSc nodules (24). The nodular structures in HSc have thin fibrils embedded in more interfibrillar matrix (24), which may impart the rigidity observed in HSc tissue.

Biglycan is a second PG found in normal skin albeit in lower quantities than decorin. Schonherr et al. (92) used immunohistochemistry to demonstrate that decorin is present throughout the normal dermis but is absent in the epidermis, and there is more decorin in the papillary zone near the dermal-epidermal junction than in the reticular dermis. In contrast, biglycan was detected only at the dermal-epidermal junction. Garg et al. (93) compared PGs from normal scar and HSc. Normal skin and normal scar contained mainly decorin with approximately 10% biglycan relative to decorin. Conversely, HSc contained about 30% biglycan which may prevent normal scar formation by some as yet unknown mechanism. Decorin is also capable of binding to TGF-β (94), type VI collagen (95), and inhibits fibroblast adhesion to fibronectin (96). Scott and coworkers (64) reported that HSc had only 25% of the decorin level of normal skin and sixfold higher levels of versican and biglycan. Versican is a large chondroitin sulfate proteoglycan, which is able to carry about 15-30 GAG chains on its protein core. Both biglycan and large chondroitin sulphate proteoglycans related to versican are normally found in articular and fibrocartilage and their hydrophilic nature may lead to an over-hydrated ECM (fig. 4). As the HSc resolves, the scars become flatter and softer and decorin levels return almost to normal. The low content of decorin in the scars is reflected in the reduced capacity of fibroblasts cultured from this tissue to synthesize the proteoglycan (97). It was recently shown by *in situ* hybridization that massive re-expression of decorin occurs in healing burn scars between about 12 and 36 months after injury (98). This is the time at which many hypertrophic scars undergo spontaneous resolution (99), leading to the suggestion that the reappearance of decorin might not only

be a secondary manifestation of scar remodelling but may contribute to maturation of the scar.

C. Fibronectin

Fibronectin is a glycoprotein which functions in the adhesion of cells and macromolecules to the ECM (100). Because of its specific functional domains and cell-binding sites, fibronectin is able to interact with a variety of cell types and function as a chemotactic agent for inflammatory cells, a scaffold for fibroblast migration and ECM deposition, and a regulator of cell growth and gene expression. Fibronectin has been reported to bind TGF-β (101) and an increase in fibronectin has been measured in HSc (102). The ability of fibronectin to induce cell migration and ECM synthesis suggests that its persistence may be involved in the development of fibrosis.

D. Elastin

In normal skin, there exists a random organization of collagen and a network of elastin which gives skin its strength, elasticity, and flexibility. In contrast, mature scars have large parallel-organized collagen bundles and a scattered elastin network (103). It has been suggested that there is a temporary absence of elastin from hypertrophic scars, which contributes to their hardness and inelasticity. De Vries et al. (104) used a human punch biopsy wound model and found that elastin-coated native collagen matrices made from insoluble collagen fibers, stimulated the formation of ECM consisting of mature collagen fibers, reduced fibroblast and myofibroblast accumulation and wound

contraction was minimal (105). In contrast, matrices coated with hyaluronic acid and fibronectin stimulated wound contraction.

It has been suggested that fragments of altered elastin fibers in injured tissue may be partially responsible for the chronic inflammatory response of immature HSc, and the altered fibers may interfere with the production of new elastin fibers (106). As the scar matures, elastic fibers return (24). Compton et al. (107) reported that elastin fibers are detected only years after wounding and that their appearance is one of the final events in healing.

IV. FIBROGENIC GROWTH FACTORS

Cells communicate with each other through the specific binding of cytokines and growth factors with protein receptors on their cell membranes. The functions of growth factors are diverse and include stimulation or inhibition of cell proliferation, differentiation, migration, or gene expression depending on the cell type involved (108). Of the many growth factors and cytokines potentially involved in HSc and keloids, Transforming growth factor-β (TGF-β) is certainly one of the most complex and pleiotropic. Because of the many functions of this growth factor, its regulation is considered crucial in the control of normal wound healing.

A. Transforming Growth Factor-β1

TGF-β belongs to a supergene family consisting of three groups, the TGF-βs, the activins and the bone morphogenic proteins (BMPs). Five isoforms of TGF-β have been identified to date; TGF-β1, -β2, -β3, -β4, -and β5. Of these, TGF-β1, -β2, and β3 are found in mammals (109). TGF-β is released from platelets into the wound environment following injury and acts as a chemotactic agent for neutrophils, T lymphocytes, monocytes and fibroblasts (109,110). Although TGF-β is essential for normal wound healing (111,112), overexpression or persistent expression of this growth factor may lead to fibrosis as seen in HSc and keloids.

TGF-β1 is implicated in the formation of HSc and keloids because of its ability to elicit an over-production of collagen. This is achieved both by upregulation of collagen synthesis and downregulation of collagenase production (113,114). It has been reported that TGF-β1 mRNA expression is greater in post-burn HSc relative to that of normal tissue obtained from the same patients (115). It has also been shown that TGF-β1 is capable of upregulating its own receptor expression (116,117) and stimulating the differentiation of fibroblasts into myofibroblasts (118).

TGF-β1 is secreted as a small latent complex (LTGF-β1) consisting of a 25 kd dimeric mature protein and an N-terminal pro-protein called the latency associated peptide (LAP) (119). Important features of the LAP are the presence of three N-linked oligosaccharides, two of which include mannose-6-phosphate (M6P) (120). In cells such as fibroblasts, platelets, and bone cells, the LTGF-β1 complex may form a large latent

complex with latent TGF-β1 binding protein (LTBP), a 125-205 kDa glycoprotein which is required for the secretion and targeting of TGF-β1 in some cells (121). The binding of LTBP masks the M6P moieties on LAP and prevents the uptake of LTGF-β into lysosomes. After its release from degranulating platelets, TGF-β1 can exist as the small latent complex and be sequestered in the ECM (122), or as the large latent complex and either be released into the serum (109) or be bound to the ECM where it can be released by proteolytic cleavage. It is generally believed that either a conformational change of the latent complex or dissociation of LAP is required for activation of TGF-β1 (123,124) as the TGF-β receptors do not recognize LTGF-β1 (125).

Wakefield et al. (126) studied the tissue distribution of both recombinant latent and active TGF-β1 in rats. Active TGF-β1 was shown to accumulate in the lungs, liver and kidney which is similar to the tissue distribution of α2-macroglobulin (127,128), which is a carrier molecule involved in the clearance of active TGF-β1. Conversely, latent TGF-β1 did not accumulate in any one organ, instead, it was present in low levels in all organs, the authors suggested that the LAP may extend the half-life of TGF-β1 in circulation by preventing it from complexing with α2-macroglobulin. Thus, while active TGF-β1 may act locally in an autocrine or paracrine fashion, latent TGF-β in circulation may have endocrine activity.

Transforming growth factor-β1 has been shown to inhibit T and B lymphocyte proliferation and differentiation (129, 130). Mice with a targeted disruption of the transforming growth factor gene (*Tgfb1-/-*) have been shown to experience multifocal

inflammatory disease with massive infiltration of lymphocytes and macrophages in most organ systems (131-133). Mice usually die at approximately 3 weeks of age. Crowe et al. (134) prolonged the life span of *Tgfb1-/-* mice by using *Scid -/-* mice which lack T and B cells and wound healing studies were performed. It was found that *Tgfb1-/- Scid -/-* mice experienced a delay in angiogenesis and cellular infiltration which did not begin until day 14 compared to day 5 in *Scid -/-*control mice. Also, the granulation tissue in *Tgfb1-/- Scid -/-* mice was thinner and less organized and apoptosis was delayed compared to *Scid -/-* controls.

Dickson and colleagues (135) used ^{125}I-TGF-β1 to demonstrate the distribution of administered active TGF-β in mice and rats. The investigators showed that the microvascular endothelium was the major site of TGF-β binding. In response to tissue injury, TGF-β upregulates adhesion molecules and has chemotactic properties. However, it has previously been suggested that a major function of systemic TGF-β may be to reduce adhesiveness of endothelial cells for immune cells by inhibiting E-selectin expression (136-139). This function is perhaps best demonstrated by MRL/1pr mice, a murine autoimmune model used to study diseases such as systemic lupus eryththematosis. In these mice, the TGF-β1 gene is disrupted and an inflammatory response results in death 2-3 weeks after birth (131,132). Whereas increased local production of TGF-β1 may result in fibrotic disorders by activating fibroblasts, endocrine TGF-β1 interacts mainly with endothelial cells (130), and to a lesser degree fibroblasts and macrophages (140,141). Chronic inflammation can lead to excessive systemic TGF-β as a control mechanism to dampen the immune response. Use of TGF-β as an

immunosuppressant has been suggested however, excessive TGF-β may lead to an unresponsive immune system resulting in life-threatening bacterial infections. Elevated endocrine TGF-β has been noted in conditions which result in immunosuppression such as SLE, HIV and arthritis (142-144).

The mechanisms by which TGF-β1 activation occurs *in vivo* have not been fully elucidated. Plasmin is capable of activating TGF-β1 by cleaving LAP (145-148). Plasmin is also the major fibrinolytic enzyme involved in wound healing and it is activated from its precursor form plasminogen, by urokinase type plasminogen activator (u-PA) and tissue type plasminogen activator (t-PA). Activation of plasmin is inhibited by plasminogen activator inhibitor-I (PAI-I). TGF-β1 itself is capable of regulating plasmin activation, and thus, potentially at least, of controlling its own activation, by upregulating PAI-I. Tuan et al. (149) reported a decrease in uPA and an increase in PAI-I levels in keloid fibroblasts versus normal fibroblasts suggesting a decrease in the role of keloid fibroblasts in fibrinolysis. This same pattern was shown after treating normal fibroblasts with TGF-β1. Another function of plasmin is the activation of matrix metalloproteases such as collagenase, which is crucial in wound remodeling (150). TGF-β1 also has a role in the regulation of matrix metalloproteinases by stimulating the synthesis of tissue inhibitor of metalloproteinases 1 (TIMP-1) (151) and downregulating collagenase mRNA (114).

Mast cell chymase, in contrast to plasmin, is released as an active-heparin bound enzyme that is not easily inhibited by protease inhibitors (152-156). Chymase releases

TGF-β1 from the extracellular matrix as a large latent complex 10-fold more efficiently than plasmin, but is not directly involved in TGF-β1 activation. However, chymase does allow for exposure and subsequent activation of latent TGF-β by other factors.

The mannose-6-phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor may be involved in the activation of LTGF-β1. Dennis and Rifkin (157) have demonstrated the binding of the small latent complex to the M6P/IGF-II receptor via the two mannose-6-phosphate moieties on the LAP. Exogenous M6P and anti-M6P receptor were able to inhibit the activation of LTGF-β1 in bovine aortic endothelial and smooth muscle cells in coculture. However, neither M6P nor anti-M6P had any effect on basal cell migration, the activity of exogenously added TGF-β1, the activation of LTGF-β1 by plasmin, or the release of LTGF-β1 from cells. Ghahary et al. (158) have studied the mechanism of TGF-β1 activation via the M6P/IGF-II receptor in a coculture system, and found that latent TGF-β1 released from genetically modified keratinocytes is capable of increasing collagen expression from dermal fibroblasts. This effect was inhibited in a dose-dependent manner by the addition of mannose-6-phosphate. This study also suggested that activation of TGF-β1 is due to a conformational change rather than due to cleavage of LAP from mature TGF-β1. Isolated fibroblast cell membranes were incubated with either latent TGF-β1 or latent TGF-β1 and recombinant active TGF-β1. Using the mink lung epithelial cell growth inhibition assay, a standard assay for demonstrating TGF-β1 bioactivity, it was shown that after centrifugation, supernatants from latent TGF-β1 alone did not significantly inhibit cell growth compared to those incubated with active TGF-β1. These results suggest that interaction of latent TGF-β1

with the M6P/IGF-II may not result in cleavage of LAP from mature TGF-β1. Although the precise mechanism by which M6P/IGF-II receptors are involved in LTGF-β1 activation is unknown, activation does require PA and plasmin (147,159). It has been proposed that the effective concentrations of both enzyme and substrate are increased by binding to the cell surface thus facilitating the activation and release of LTGF-β1.

In vitro studies have suggested a role for retinoids in the activation of TGF-β1 through their ability to increase plasminogen and plasmin levels and to increase the expression of cellular type II transglutaminase (160). Transglutaminase has been shown to be required for TGF-β1 activation, possibly by concentrating plasminogen activator (PA) to the extracellular matrix by cross-linking it to fibronectin (161). Thrombospondin is a glycoprotein that is also capable of activating both the large and small latent complexes of TGF-β but without proteolytic cleavage of LAP from TGF-β1. Instead, it may work by inducing a change in conformation (162). Similar to thrombospondin, it has been suggested that IgG may also be capable of activating TGF-β independent of proteases (163). Active TGF-β1 in MRL/1pr mice was found complexed to IgG in B cells and plasma cells. This complex was shown to strongly inhibit neutrophil function by inhibiting the adhesion and subsequent uptake of bacteria to activated neutrophils. The IgG-TGF-β complex was shown to be 500 times more potent than recombinant active TGF-β in suppressing neutrophil function. This may be due to a more efficient presentation of active TGF-β1 to neutrophil TGF-β receptors by IgG or because IgG functions as a carrier molecule thus extending the half-life of active TGF-β in circulation (163).

Once TGF-β1 is activated, it is capable of binding to heteromeric receptor complexes consisting of type I (RI) and type II (RII) receptors. Each of these receptors possesses a serine/threonine kinase and both receptors are required for signal transduction following TGF-β1 binding. Receptor type II is necessary for the recruitment and activation of RI (164), and RI is responsible for the propagation of the signal to downstream targets (165,166). In normal human skin, RI and RII are present in the epidermis, epidermal appendages, and in vascular cells. Schmid et al. (167) reported that in granulation tissue, the expression of both receptors increased and, as remodeling proceeded, the levels decreased. However, in HSc, the levels of both RI and RII remained high for up to 20 months after injury. It was proposed that the failure to clear high receptor-expressing fibroblasts during remodeling induced a positive feedback loop for the autoinduction of TGF-β1. TGF-β1 is capable of autoinducing TGF-β1 mRNA transcription via activation of the AP-1 complex consisting of *c-jun* and *c-fos* proto-oncogene proteins (168). High levels of the cytokine may thus persist long after the initiating stimulus and this may contribute to the development of fibroproliferative disorders.

The downstream molecules responsible for TGF-β1 signal transduction are able to produce diverse cellular responses following TGF-β1 binding to its receptor. TGF-β1 is both a stimulatory and an inhibitory molecule. It is a chemoattractant for monocytes, neutrophils and fibroblasts and induces the release of IL-1, IL-6, TNF-α and bFGF from these cells. (151). The effect of TGF-β1 on target cells depends on many factors

including cell origin, the state of differentiation, local concentrations of activating and inhibiting molecules and the presence of other growth factors and cytokines (151).

The half-life of active TGF-β is approximately 2-3 minutes and yet physiological levels are maintained at about 5 ng/mL in normal humans, indicating that carrier proteins may be involved in transporting TGF-β in the plasma (169). Thrombospondin, IgG, or α2-macroglobulin may act as carrier molecules for latent TGF-β (126).

Once activated, regulation of TGF-β1 appears to occur by its binding molecules such as the proteoglycan decorin, in the ECM and α2-macroglobulin in the circulation. Mast cells may enhance the levels of TGF-β1 as heparin is capable of releasing active TGF-β1 from α2-macroglobulin (170). It has been reported that patients with HSc and keloids have a statistically significant increase in allergy symptoms (60) which are often associated with an increase in IgE levels and mast cells counts (171). The decrease in decorin content in HSc (discussed above) may be in part due to TGF-β1. Scott et al. (172) used normal and HSc fibroblasts from the same patients to show that decorin synthesis was lower in HSc compared to normal fibroblasts and following TGF-β1 treatment, decorin was further reduced in all six strains of HSc and in five of the six strains of normal fibroblasts. After removal of TGF-β1 and passaging cells, decorin synthesis was no longer suppressed. The decrease in decorin following TGF-β1 treatment is in agreement with the results of Kahari et al. (173) who treated normal human skin and gingival fibroblasts with TGF-β1. Proteoglycans may normally function to control cell proliferation by regulating growth factors such as TGF-β1 and bFGF in the

ECM (174,175), or conversely, downregulation of decorin expression in HSc by TGF-β1 may be associated with the increased cell numbers involved.

The localization of decorin, versican, biglycan and TGF-β has been demonstrated in normal skin, mature scars, and HSc (176). In normal skin, decorin was present throughout the dermis, versican and biglycan were present in very low levels and TGF-β1 was not detected. In HSc, decorin was present in the deep dermis and a narrow zone under the epidermis but was absent in the ultrastructural nodules typical of HSc; whereas, TGF-β1 was localized to the nodules and the deep dermis. Scott and collegues proposed that the co-localization of TGF-β1 and decorin in the deep dermis may be important in the resolution of the scar as staining for both was quite intense in this region in the mature scars.

B. Insulin-like Growth Factor-1

IGF-1 is another growth factor that may promote excessive matrix deposition in HSc and keloids due to its mitogenic effects (177), its ability to stimulate synthesis of certain PGs (178), and collagen by fibroblasts (179). Insulin-like growth factors are expressed in most tissues at various stages in development and may function as autocrine, paracrine or endocrine factors (180). In the uterus, IGF-1 is mainly regulated by estrogen (181). Estrogen is involved in the proliferation of many uterine cells such as stromal and epithelial cells resulting in uterine growth (182). It has been shown that IGF-1 mRNA (183) and the IGF-1 receptor (184) expression are increased following estrogen treatment. Estrogen has also been shown to down-regulate insulin-like growth factor

binding protein (IGFBP)-1, a binding protein capable of inhibiting the growth-promoting effects of IGF-1 (185). There may be an estrogen-responsive element in the IGF-1 gene, which interacts with an activated estrogen receptor. In rats, estrogen has been shown to inhibit the expression of IGF-1 mRNA in tissues such as kidney, lung and liver (186). However, recent studies in humans and primates indicate that low doses of estrogen may stimulate growth in other tissues (187-189), perhaps through enhanced growth hormone secretion (190).

IGF-1 in the serum is bound to specific binding proteins which protect it from proteolytic degradation (191,192). Type III collagen and fibronectin are capable of binding IGFBP-3 and -5 (192), so that IGF-1 released from immune and epithelial cells may associate with the ECM. IGF-1 may contribute to the development of HSc due to its ability to increase mRNAs for type I and type III procollagens and down regulate collagenase activity (114,179). Ghahary et al. (179) have demonstrated an approximately 2-fold increase in IGF-1 mRNA in HSc compared to normal dermis from the same patients. Treating dermal fibroblasts with IGF-1 was associated wth a 150% increase in pro α1(I) mRNA and a 170% increase in pro α1(III) mRNA.

IGF-1 levels in HSc could be increased by the disruption of sweat and sebaceous glands following injury (193). In normal skin, IGF-1 is localized to the epithelial cells located in the superficial epidermal layer, sweat and sebaceous glands, and in the deep dermis. However, in HSc, these structures are disrupted. Re-epithelialization is dependent upon deep dermal epithelial cells migrating from the residual sweat and

sebaceous elements where they are able to secrete IGF-1 in the presence of dermal fibroblasts. As these cells contribute to re-epithelialization and to the healing of sweat and sebaceous glands in the skin, the fibroblasts may no longer be exposed to IGF-1. This could facilitate the resolution of HSc. Interestingly, animals such as the rat, rabbit, mouse and pig lack sweat glands similar to those seen in humans and do not develop keloids or HSc.

IGF-1 may also be capable of inducing TGF-β1 expression in dermal fibroblasts thus augmenting the fibrotic environment. These growth factors are co-expressed in several physiological and pathological conditions by different cell types such as fibroblasts (194,195), platelets (8,196), and activated macrophages (196,197). Ghahary et al. (198) reported that treatment of fibroblasts with IGF-1 caused an increase in transcription of TGF-β and protein production and this effect persisted for at least 48 hours after withdrawing IGF-1. It was proposed that IGF-1 may stimulate the expression of TGF-β1 mRNA in dermal fibroblasts through activation of the AP-1 complex. Transforming growth factor-β1 may then act as an autocrine factor and induce its own further expression.

V. EMERGING THERAPIES

Controlling excess ECM deposition appears to be the key in preventing HSc and keloids. This could be achieved either by controlling the deposition of collagen or by increasing the activity of collagenase and thus promoting collagen degradation.

Interferons have been shown to be potent antifibrotic factors and recently, the peptide hormone relaxin has also been considered as a candidate for the management of fibroproliferative disorders.

A. Interferons

Interferon-α, -β, and -γ were originally identified on the basis of their antiviral activity. IFN-α and -β are produced by almost all nucleated cells and IFN-γ is produced by activated T lymphocytes. Interferons bind to high affinity receptors, each of which is associated with two tyrosine kinases from the Janus family. These kinases phosphorylate cytoplasmic signal transduction proteins that are then capable of enhancing or inhibiting the transcription of various genes (199). All three interferons are capable of decreasing the synthesis of types I and III collagen (200,201), inhibiting fibroblast proliferation and controlling cytoskeletal protein-mediated wound contraction. IFN-α2b may also function to reduce wound cellularity in the later stages of healing by inducing apoptosis of fibroblasts (202). The effect of IFN-α2b in a model of wound contraction was demonstrated by Nedelec et al. (203), whereby collagen lattices were seeded with fibroblasts from matched tissue samples of human HSc and normal dermis. Treatment with IFN-α2b decreased the rate and degree of contraction by both normal and HSc fibroblasts. This process involved a lag phase indicating that protein synthesis was required (204-206). Although others have reported an increased ability of HSc fibroblasts to contract these lattices compared to normal fibroblasts (207), Nedelec et al. (203) reported similar rates of contraction. The mechanism by which IFN-α2b inhibits contraction may be through reduction in mRNAs of β- and γ-actin, as actin filaments are

required for fibroblast elongation and contraction (34,38,208,209). In addition to the reduction in actin mRNA, Nedelec and coworkers also noted changes in the organization of microfilaments, the bipolar morphology characteristic of contractile cells was lost following IFN-α2b treatment.

Interferon-α2b may also modulate the effects of fibrogenic growth factors. IGF-1 is expressed in higher levels in HSc than normal dermis (179), where it acts as a mitogen (210). Recently, it was shown that IFN-α2b suppresses the fibrogenic effects of IGF-1 (211). Human fibroblasts grown in culture were treated with IFN-α2b and IGF-1 and showed a 44% decrease in hydroxyproline (an index of collagen protein) and an approximately 4-fold increase in collagenase activity over cells treated with IGF-1 alone. An important distinction between IFN-α2b and IFN-γ is that IFN-α2b is capable of increasing expression of collagenase while IFN-γ decreases collagenase activity (212).

Interferons are also capable of inducing the production of nitric oxide by fibroblasts. Nitric oxide (NO) is an intracellular messenger molecule which may have roles in immunoregulation and inflammation (213). Nitric oxide can be released from phagocytes, hepatocytes and in cartilage (214,215). Its effects include: prevention of platelet aggregation (216), inhibition of histamine release from mast cells (217), and increase in metalloproteinase activation. Metalloproteinases such as collagenase and stromelysin are synthesized as proenzymes and require activation in the ECM. Murrell et al. (218) showed that in the presence of inflammatory mediators such as interleukin-1β and tumor necrosis factor-α, both nitric oxide synthase and metalloproteinase activity

were increased in explants of bovine and human cartilage. It has been reported that fibroblasts from HSc produce less NO than those from normal dermis (219), and may therefore, have a diminished ability to activate collagenase. Wang et al. (220) reported that fibroblasts from normal skin produced NO constitutively and after induction following exposure to IFN-γ and lipopolysaccharide. Nitric oxide is also a vasodilator and could cause an increase in blood flow and migration of cells to the site of injury.

The use of IFN-α2b *in vivo* has had promising results in fibroproliferative disorders. Berman et al. (221) studied the recurrence rates of excised keloids treated with IFN-α2b and triamcinolone acetonide (TAC) injections. Surgical removal of keloids is normally associated with a recurrence rate of 45-100% (53,200). Berman et al. reported a 51.2% recurrence of excised lesions with no treatment, 58.5% recurrence of TAC treated lesions, and only an 18.7% recurrence of IFN-α2b-treated keloids. Since IFN-α, and -β enhance keloid collagenase activity, intralesional injection of IFN-α2b may be capable of increasing collagen breakdown and reducing the size of non-excised lesions (201). In fact, systemic injection of IFN-α2b may serve to compensate for an deficiency in keloid patients as decreased levels of IFN-γ and IFN-α have been reported in circulating immune cells of these patients (222).

Interferon-γ is another promising anti-fibrogenic agent, as shown by its ability to decrease collagen deposition around subcutaneously implanted foreign bodies in mice (223). Harrop et al. (224) studied matched HSc and normal skin fibroblasts *in vitro* and reported that IFN-γ (1000 U/mL) reduced types I (55%) and III (36%) procollagen

mRNA and collagen production (34%) in HSc cells, which were as sensitive to IFN-γ as the normal fibroblasts.

A clinical study using both IFN-α2b and IFN-γ has recently been reported by Tredget et al. (225). Patients with HSc were treated with subcutaneous IFN-α2b. Punch biopsies and blood samples were taken before, during and after treatment. Dermal fibroblasts were isolated from the explants. IFN-α2b and -γ treatment of normal fibroblasts inhibited the proliferation of the cells in the absence of TGF-β1. TGF-β1 treatment of both normal and HSc fibroblasts was found to increase proliferation and collagen production of both cell types in a dose-dependent manner which was antagonized by IFN-α2b and -γ when administered separately and in combination (a weak additive effect was noted). Both interferons also inhibited the amount of TGF-β1 mRNA and protein synthesized by both normal and HSc fibroblasts. Serum samples from the patients indicated higher levels of circulating TGF-β1 than a pool of normal individuals and treatment with IFN-α2b significantly reduced TGF-β1 levels from HSc patients into the normal range during and shortly after treatment. IFN-α2b also decreased TGF-β1 mRNA in HSc tissue into the normal range within one month. Another clinical study involved administering recombinant IFN-α2b subcutaneously to 9 patients with HSc (226). These patients initially had elevated serum TGF-β1 levels relative to normal controls, had significantly elevated plasma N^{τ}-methylhistamine, the stable metabolite of histamine, and 2-fold increases in mast cell numbers in the resolving scar. With systemic IFN-α2b, N^{τ}-methylhistamine levels decreased to normal without significant changes in mast cell numbers suggesting a reduction in mast cell degranulation. Mast cell chymase

is capable of activating matrix-bound TGF-β1 (59) and histamine stimulates collagen synthesis and crosslinking (57,227).

B. Relaxin

Relaxin is a hormone of ovarian origin that is involved in pregnancy and parturition by inducing collagen remodeling by an unknown mechanism. Relaxin is a member of the same family as insulin and insulin-like growth factors (228). It is similar to the interferons in that it is capable of stimulating nitric oxide (NO) production (216).

Vakilenko et al. (229) demonstrated the growth-promoting effects of relaxin on the uterus, cervix, and vagina of rats and found that relaxin decreased total collagen and increased total glycosaminoglycans. The net result was an increased growth of the uterus, cervix and vagina by increasing water content and tissue mass. A model proposed by the authors was that relaxin increases the distensibility of the collagen matrix of the uterus.

C. Anti-Transforming Growth Factor-beta

Fetal wounds heal without scarring and have a lower inflammatory and cytokine response compared to adults (230,231). Administration of TGF-β to fetal wounds induces scarring (232). Suppression of TGF-β in adults by using antibodies, which target TGF-β has been proposed as a possible therapy to reduce scar formation. Shah et al (233,234) used an adult rat dermal wound model to demonstrate that anti-TGF-β1,2 administration at the time of wounding or shortly after resulted in a dose-dependent

reduction in scarring. The wounds treated with anti-TGF-β1,2 had fewer macrophages, monocytes, and blood vessels than control wounds. The anti-TGF-β1,2 wounds also had reduced type I and type III collagen and fibronectin levels, but retained the same tensile strength as controls. The similarity in wound strength in the anti-TGF-β1,2 wounds, despite the lower collagen content, was considered to be due to the regular arrangement of the fibrils in these wounds compared to abnormally oriented collagen fibrils in wounds treated with TGF-β-irrelevant antibody, or no injection (233). The authors suggest that the reduction in TGF-β immediately after wounding helps prevent scarring by decreasing the recruitment of immune cells. It may also alter levels of PDGF, bFGF, as well as the autocrine induction of TGF-β. Early administration of anti-TGF-β may also decrease the synthesis of PAI-1 and increase the synthesis of plasminogen and plasmin, which aid in fibrinolysis as ECM production ensues. This may result in a more organized pattern of the ECM proteins.

D. Mannose-6-Phosphate

Another potential therapy for the prevention of fibroproliferative disorders is exogenous mannose-6-phosphate (M6P). As previously discussed, latent TGF-β binds to the M6P/IGF-II receptor and its activation is inhibited by the addition of M6P (120,157) or antibodies directed against this receptor (157). It has not been determined whether cell surface-associated plasmin alone activates latent TGF-β after it binds to the M6P/IGF-II receptor (157), or whether latent TGF-β is internalized and the low pH in the endosomal compartment is responsible for activation (235). Although further investigation is

required to determine potential side effects of administration, it is reasonable to consider M6P as a therapy for excessive scarring.

VI. GENE THERAPY

Current standard therapies for the treatment of HSc and keloids have had limited success. Surgical excision without adjuvant therapy is associated with a high rate of recurrence (53,200). HSc and keloids are the result of a variety of cellular and molecular processes that are disrupted. By understanding the growth factors and cellular processes involved, therapies can be designed which either provide factors, which are diminished in the pathological condition or conversely, provide factors to regulate wound healing and prevent excess scaring.

New recombinant DNA technologies have increased the availability of growth factors and also furthered our understanding of their functions. Clinical application of growth factors has gained considerable interest. However, the use of recombinant proteins is limited because of their relative expense and often very short half-lives (126,236). The goal of gene therapy is to transfer the gene of interest into specific cells where it will direct the synthesis of recombinant protein. This form of therapy may have several advantages over the direct administration of recombinant proteins. Proteins made by the host may be more likely to be sustained at therapeutic concentrations and frequent injections would not be required. Gene delivery may be achieved either *ex vivo* or *in vivo* (237). *Ex vivo* gene therapy involves the isolation of cells from a biopsy before

transplanting them back into the host. This technique is limited to those cells that are amenable to cell culture and transplantation but has the advantage that conditions of genetic modification can be controlled. The *in vivo* technique involves delivering the gene directly into the tissue. One potential disadvantage of this method is the accuracy required for targeting the appropriate tissue.

Although non-viral methods such as liposomes and particle-mediated gene therapy give lower transfer efficiencies than viral methods, these methods can yield positive results. Liposomes consist of one or more lipid bilayer membranes with fatty acid tails on the interior and hydrophilic heads facing exterior, exposed to the aqueous phase. These bilayers form aqueous compartments and the lipid composition and preparation technique determines the size and shape of the vesicle. Targeting of the liposome may be achieved by inserting monoclonal antibodies into the outer membrane (238,239). The skin is amenable to gene therapy because of its accessibility. Liposomes can deliver their contents to the skin, either by dehydration of the liposome in the stratum corneum (240) or by penetration of the skin through hair follicles (241,242).

Early liposomes were made with phosphatidylserine, which has a net negative charge (243-245). For the lipid-DNA interaction to occur, DNA first had to be encapsulated by reverse phase evaporation using phosphatidylserine and cholesterol. A more efficient method of DNA incorporation, termed "lipofection", was later developed by using cationic lipids which interact with negatively charged strands of DNA. The complex is then taken into the target cell either by fusing with the plasma membrane

(246-248) or through endocytosis, following which the DNA may escape from either early or late endosomes into the cytoplasm (248-250). Cationic lipids transfect different cell types with varying efficiencies and the level of toxicity may also vary with cell type (246,251-258). Cationic liposomes have been used to deliver plasmid DNA, RNA (259) and protein (260,261).

The advantages of lipofection are that the size of the gene to be delivered is not limited, they are easier to prepare and test compared to viral constructs and they are relatively non-toxic. However, this method is associated with a low frequency of stable transfection, 100-1000 times more DNA is required for liposomal delivery compared to adenoviral constructs (262). Thus far, liposomes are the only non-viral gene transfer method being tested in clinical trials.

Particle-mediated gene transfer uses microparticles such as gold or tungsten coated with DNA. The particles are then used to bombard the cells or tissue at a high force in order to penetrate the cell membrane and deliver the DNA to the cytoplasm. However, in addition to the possibility of damaging the cell membrane, the transferred genes are expressed transiently and the frequency of stable integration is low (263). The advantages of this method are its applicability to *in vivo* gene transfer, the capability of transferring large DNA molecules, and the ability to use it on a variety of cell types since the cell membrane is unable to act as a selective barrier.

Viral vectors provide the most efficient methods for gene transfer to date. Replication deficient recombinant viral particles may be used for gene transfer into human cells. Retroviruses used for gene therapy are single-stranded RNA viruses approximately 8 kilo basepairs (kbp) in size. They are made replication deficient by deleting all viral sequences except those required for packaging RNA into the virion, integration of viral DNA into the genome, and expression of proviral encoded proteins (264-266). These deleted sequences are replaced with the desired gene (267). Packaging cells replace those functions that are deleted. The packaging cells shed the viral particles into the medium, which is then incubated with target cells. The virus adsorbs onto the target cell using specific receptors or enters via endocytosis (268). Once the viral envelope fuses with the target cell, viral RNA enters the cytosol where it is reverse-transcribed into double stranded DNA before it is randomly integrated into the host cell genome (264,265,267). Retroviruses are capable of stable integration in a variety of cell types with minimal rearrangement, however this method is limited to the size of gene that can be transferred (less than 6 kbp). A further disadvantage of this method of gene transfer is that integration of most retroviruses (HIV being the exception) only occurs in dividing cells during nuclear breakdown (269,270). Integration is limited by the rate of intracellular decay of the retrovirus such that only viruses that enter cells shortly before division are able to integrate into the host genome. There is current research underway using a retroviral vector based on a lentivirus that is capable of integrating into the genome of non-proliferating cells (271). Other disadvantages include the possibility of insertional mutagenesis, possibly of a tumor suppressor gene, resulting in tumorigenesis. The retrovirus might also recombine with a replication-competent virus (272).

Recombinant adenoviruses are double stranded, non-enveloped viruses containing DNA, approximately 36 kbp in size. The use of adenoviral gene transfer is relatively safe following the deletion of genes required for replication and cellular transformation. Deletion of viral genes is also necessary to accommodate the gene of interest since the size of the viral genome cannot exceed 105% in order to be packaged into virion procapsid (273). New techniques are being developed whereby adenoviruses act as carriers for DNA (274). The adenovirus can attach to and penetrate the cell via coated pits. Once inside the endocytic vesicles, the adenovirus causes lysis of the vesicle before degradation occurs. The advantage of this method of gene transfer is that larger DNA molecules may be used than if the DNA were inside the viral capsid. However, this method is less efficient than when the gene is inside the capsid and the complexes can aggregate, which may result in toxicity.

This form of gene delivery demonstrates a broad host range, the highest gene transfer efficiency *in vivo* and these viruses can infect both dividing and non-dividing cells with high efficiency. In contrast to retroviruses, which may cause insertional mutagenesis, adenoviral replication occurs outside the nucleus. However, possible disadvantages of this method include an inflammatory response to viral particles and the transient gene expression which may be due to a dilution effect as cells divide, degradation of vector DNA, or to the immune response elicited by viral proteins (275,276). The inflammatory response presently prevents the repeated use of adenoviruses due to neutralizing antibodies and cellular immunity (277,278)

Although better gene transfer using viral methods can be obtained compared to non-viral methods, there are risks; including the potential of replication competent viruses and in the case of retroviruses target cell transformation by insertional mutagenesis.

The effects of fibrogenic growth factors such as TGF-β1 appear to be central in the development of HSc and keloids so therapies directed towards decreasing bioactive levels of these growth factors or correcting the ECM defects manifested by them appear to be key in controlling these lesions.

By understanding the regulation of TGF-β1, it is possible to develop methods such as the use of connective tissue growth factor antagonists, which minimize levels of active TGF-β1, but not to the point where the required effects of TGF-β1 are also lost. Decorin is able to bind and neutralize TGF-β through its core protein (279). Isaka et al. (280) had shown that injection of recombinant decorin into rats with experimental glomerulonephritis was as effective as using anti-TGF-β1 antibodies in reducing ECM accumulation in the glomeruli. This same group later used muscle-based gene therapy using the rat skeletal muscle to produce the recombinant protein. It is known that decorin is a secreted protein that when injected, it is taken up by the liver, kidney and lung (281). Decorin gene transfer was shown to decrease proteinuria in these rats, decrease glomerular TGF-β1 mRNA expression 37% lower than control (pAct-CAT transfected), and decrease ECM accumulation. This reduction may be adequate in reducing fibrosis

without an extreme reduction which is pathological (282). This study demonstrated the ability to safely deliver a protein into the systemic circulation by injecting the DNA into skeletal muscle. The potential therapeutic effects of decorin lie not only in its ability to bind and neutralize TGF-β1 but also in its role in collagen fibril organization (172).

Another therapeutic agent for fibroproliferative disorders that is being studied is IFN-α2b. Interferons are cytokines with anti-proliferative properties and it has been suggested that they would be successful in counteracting some of the processes occurring in HSc and keloids. The interferons have been shown to decrease collagen synthesis and inhibit proliferation and chemotaxis of normal human fibroblasts (224,283-285). IFN-α2b may also be able suppress the effects of IGF-1 (discussed above). The effect of Interferon-α2b was demonstrated by encapsulating it in liposomes and applying it topically to guinea pig wounds to assess its effect on wound healing (286). A significant reduction in the rate of contraction was reported after five days, which continued up to 10 days. There was also a reduction in pro-α1(I) type I collagen and pro-α1(III) mRNA. Since dermal fibrotic conditions are associated with an excess of type I and type III collagen, this finding suggests that IFN-α2b may be a successful therapeutic agent.

IFN-α has recently been considered in cancer therapy due to its antiproliferative properties (284). In particular, IFN-α has been shown to inhibit chronic myelogenous leukemia (CML) bone marrow progenitor growth (286,289). The CML progenitor cells express higher levels of FAS receptor in the presence of IFN-α and this is thought to make them more susceptible to apoptosis. Therefore over-expression of IFN-α may

inhibit CML growth but still allow normal cell growth. Adenovirus-mediated gene transfer was used to transfect IFN-α into normal human $CD34^+$ stem cells *in vitro* and the investigators found no suppression of cell growth or differentiation. This method of gene transfer may prove successful in wound healing models due to its lack of suppressive effects on normal cells and its transient expression.

VII. FIGURES AND LEGENDS

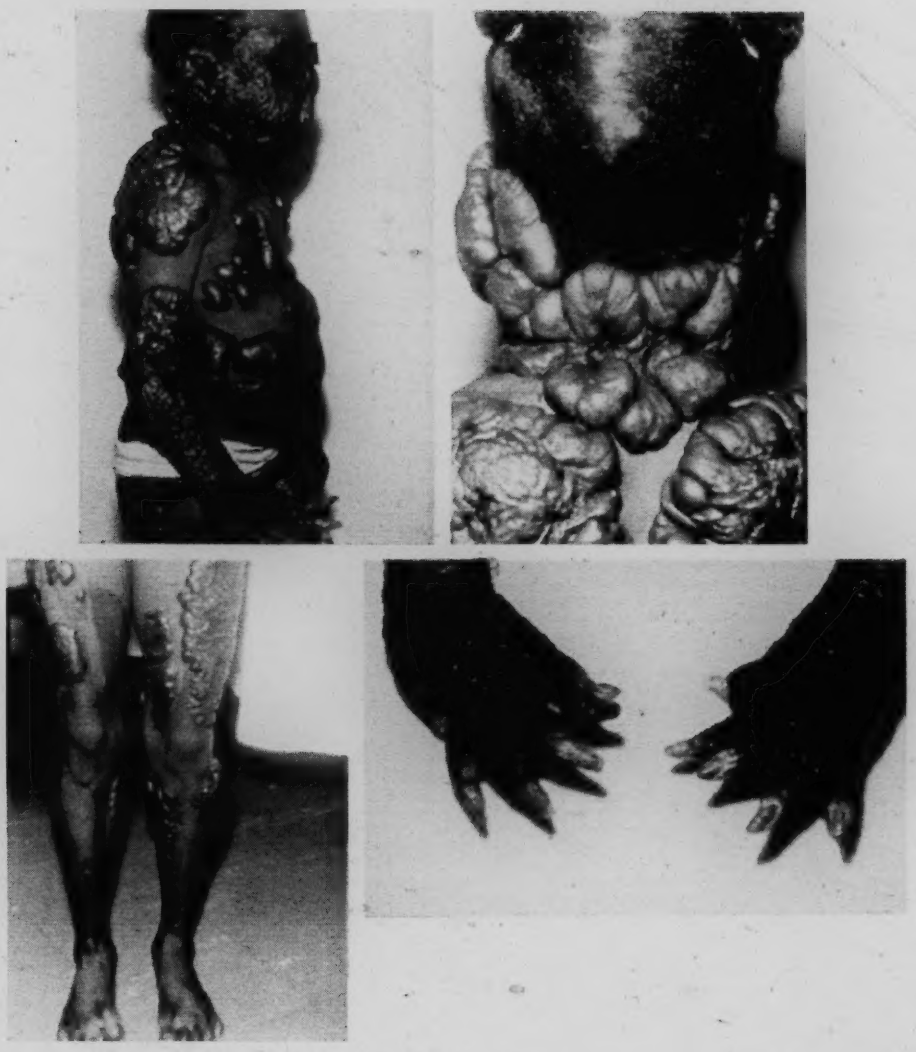

Figure 1-1

A 12-year-old black child with severe keloids following a scald injury (*From* Scott PG, Ghahary A, Chambers MM, Tredget EE. Biological basis of hypertrophic scarring. *In* Malhotra S (ed): Advances in Structural Biology. Connecticut, JAI Press Inc 1994;157. With permission).

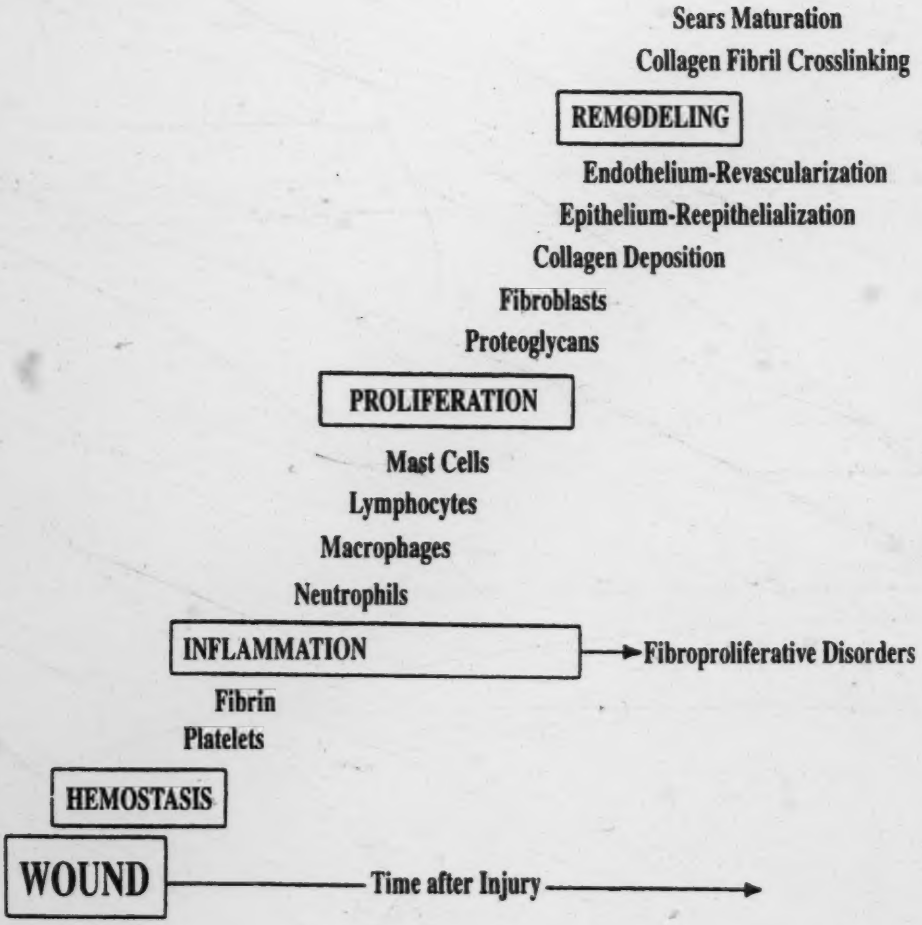

Figure 1-2

The phases of normal wound repair follow an orderly sequence of events that are regulated by the chronologic appearance of a number of different cell types over the course of healing. Prolonged activity or abnormal levels of fibrogenic cytokines released during the inflammatory phase may lead to fibroproliferative disorders (*From* Tredget EE, Nedelec B, Scott PG, Ghahary A. Hypertrophic scars, keloids, and contractures. Surg Clin N Am 1997; 77:705. With permission).

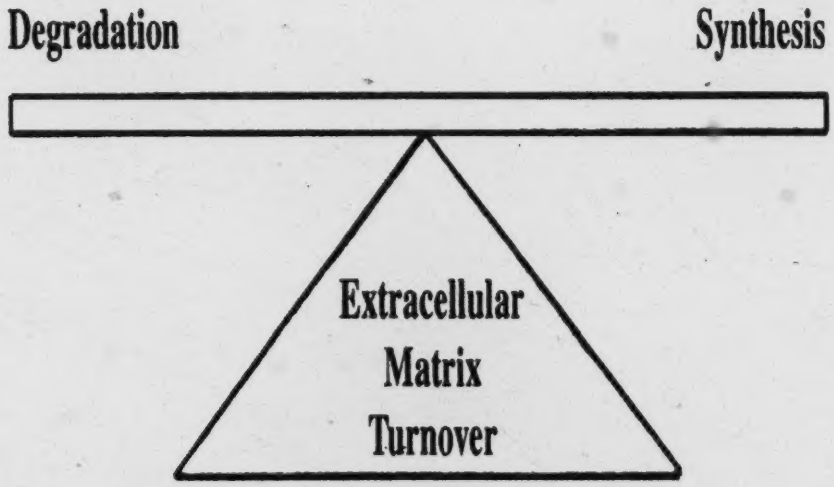

Figure 1-3

Regeneration of extracellular matrix homeostasis requires a dynamic balance between synthesis and degradation to achieve optimal wound healing (*From* Tredget EE, Nedelec B, Scott PG, Ghahary A. Hypertrophic scars, keloids, and contractures. Surg Clin N Am 1997; 77:705. With permission).

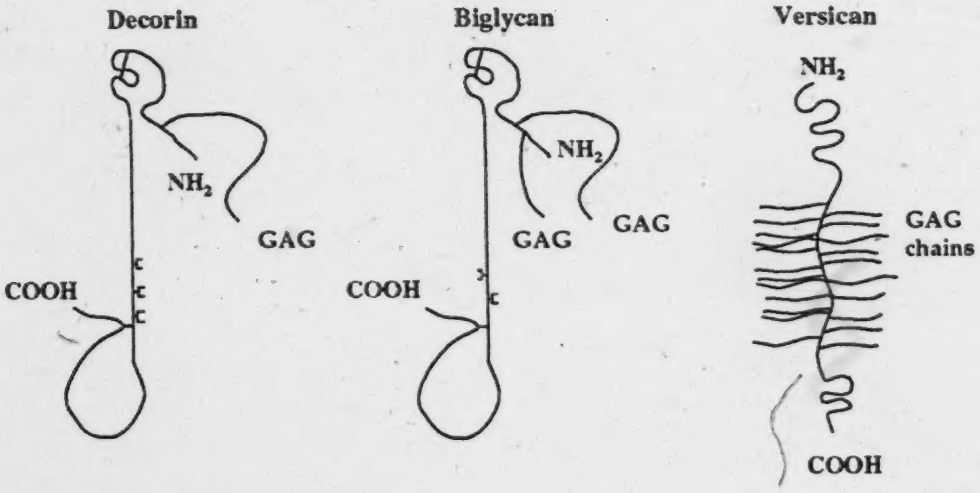

Figure 1-4

Hydrophilic sugar chains associated with the glycosaminoglycans are elevated in HSc. Decorin and biglycan are small proteoglycans with one and two dermatan sulfate sugar chains, respectively. Versican is a large proteoglycan with as many as 30 sugar chains, thereby contributing significant rigidity to HSc because of its hydrophilic properties (Not drawn to scale). (*From* Tredget EE, Nedelec B, Scott PG, Ghahary A. Hypertrophic scars, keloids, and contractures. Surg Clin N Am 1997; 77:705. With permission).

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CHAPTER 2

Latent and Active Transforming Growth Factor-β1 Released from Genetically Modified Keratinocytes Modulates Extracellular Matrix Expression by Dermal Fibroblasts in a Co-culture System

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I. ABSTRACT

Transforming growth factor-beta1 (TGF-β1) is a multifunctional cytokine involved in many aspects of wound healing. Here we report the effects of both latent and active TGF-β1 released from genetically modified keratinocytes on extracellular matrix (ECM) expression by dermal fibroblasts in a co-culture system. Human keratinocytes were genetically modified with adenovirus containing either cDNA for latent TGF-β1 (AdTGF-β1) or active TGF-β1 (AdTGF-$\beta1^{223/225}$) or LacZ and cultured with human dermal fibroblasts. Northern blotting for mRNA confirmed that keratinocytes were successfully transduced with the adenoviruses as the cDNA transcripts are smaller than native TGF-β1 mRNA. An enzyme-linked immunosorbent assay (ELISA) specific for TGF-β1 demonstrated that the TGF-β1 produced by the genetically modified keratinocytes was able to pass through the membrane separating the two cell layers. Levels of TGF-β1 were significantly higher for both latent ($p<0.0001$) and active ($p<0.0001$) TGF-β1 compared to the LacZ control. Without acid activation of samples, keratinocytes transduced with the active TGF-β1 construct exhibited significantly higher levels of TGF-β1 than either the latent construct or the LacZ control ($p<0.0001$). The TGF-β1 produced was biologically active, as shown by the plasminogen activator inhibitor (PAI/L) assay ($p<0.0001$). To demonstrate that TGF-β1 had an effect on underlying fibroblasts, mRNA was extracted and analyzed using Northern analysis. Latent TGF-β1 significantly increased the expression of type I collagen mRNA ($p<0.05$) but did not significantly affect collagenase mRNA. Active TGF-β1 significantly increased type I collagen mRNA ($p<0.005$) while also decreasing collagenase mRNA

($p<0.05$). These results illustrate the ability of increased levels of TGF-β1 to override the effects of normal keratinocytes on the behavior of dermal fibroblasts.

II. RATIONALE FOR CURRENT STUDY

The study of fibroproliferative disorders has allowed insight into the role of cells and growth factors in normal and defective wound healing. Due to the important role TGF-β1 plays in cell chemotaxis, fibroblast proliferation and wound contraction, we chose to use this growth factor in an impaired wound healing model. We obtained two adenoviral vectors, each containing either the latent or active form of TGF-β1. Our goal is to first test these adenoviral constructs *in vitro* in a co-culture system consisting of human keratinocytes and dermal fibroblasts to determine the effect of TGF-β1 on fibroblasts in the presence of keratinocytes. We then plan to use these constructs *in vivo* in an animal model of chronic wound healing. We plan to use the JCR:LA-cp rat as our *in vivo* model. This rat exhibits many similarities to the *db/db* mouse in that it is obese, insulin resistant and leptin resistant. Interestingly, the wound healing characteristics of this rat strain have not been studied to date.

III. INTRODUCTION

Transforming growth factor beta-1 (TGF-β1) is a multifunctional growth factor important in the process of wound healing due to its fibrogenic effects. Five TGF-β isoforms have been identified to date, with TGF-β1, 2, and 3 being found in mammals. During wound healing, TGF-β1 stimulates extracellular matrix (ECM) synthesis and deposition by inducing fibroblasts to synthesize collagen, fibronectin and

glycosaminoglycans (1,2). TGF-β1 also modulates the expression of proteases and their inhibitors (3,4), acts as a chemoattractant for monocytes (5) and fibroblasts (6), and enhances neovascularization (7).

Transforming growth factor-β1 is secreted as a latent complex (LTGF-β1) consisting of a 25-kDa dimeric mature protein and an N-terminal pro-protein called the latency-associated peptide (LAP) (8). It has been suggested that either a conformational change of the latent complex (9) or dissociation of LAP from the mature protein is required for activation of TGF-β1 (10,11) as the TGF-β1 receptors do not recognize native LTGF-β1 (5). Although the mechanisms by which TGF-β1 is activated *in vivo* have not been fully elucidated, plasmin and cathepsin D are capable of activating TGF-β1 by cleaving LAP from the mature protein in the N-terminal region (12-15). *In vitro*, TGF-β1 can be activated by extremes of pH and heat (80°C for 10 min) and by detergents (16). Latency is very important in the regulation of TGF-β and previous experiments have shown very different outcomes depending on the state of the TGF-β1 (17-18).

Many growth factors require prolonged exposure to the wound environment before eliciting an effect. Lynch *et al.* (19) demonstrated that in a partial thickness dermatome wound in pigs, single topical applications of EGF, TGF-α, bFGF, IGF-1 and PDGF-BB did not accelerate healing. It has also been reported that daily injections of EGF into polyvinyl alcohol sponges implanted subcutaneously into rats produced only a small increase in granulation tissue while using slow-release pellets carrying EGF resulted in a significant increase in granulation tissue volume and organization (20). The

concentration of recombinant growth factors required to stimulate DNA synthesis or cell migration *in vivo* is 1,000 times higher than that required *in vitro* (21). This may be due to proteolytic degradation and/or diffusion of the growth factor from the wound. Thus a potential solution to this problem seems to be the use of a genetically modified skin substitute through which a wound healing promoting factor such as TGF-β1 is released at the wound site. This study was conducted to achieve the following objectives. First, to examine the expression of both latent and active TGF-β1 from genetically modified human keratinocytes. Second, to demonstrate that the TGF-β1 produced was biologically active using a novel sensitive bioassay for TGF-β1 activity, the plasminogen activator inhibitor/luciferase (PAI/L) assay (22). Third, to study the effect of latent and active TGF-β1 on fibroblast proliferation and on the expression of type I collagen and collagenase in human dermal fibroblasts co-cultured with TGF-β1 genetically modified keratinocytes.

IV. METHODS AND MATERIALS

Adenoviral vectors expressing either active TGF-β1, latent TGF-β1 or LacZ as a control were used in this study. Two recombinant adenoviruses were kindly provided by Dr. Jack Gauldie (Department of Biology, Health Sciences Center, McMaster University) (18). The AdLacZ control was obtained from Quantum Biotechnologies Inc. Chaval, Quebec, Canada. Briefly, both recombinant TGF-β1 adenoviruses contain the cDNA of the coding region of full-length porcine TGF-β1 as described by Sime *et al.* (18). The cDNA fragments for latent and active TGF-β1 were 1.5- and 1.1-kb respectively. Latent TGF-β1 was expressed by the AdTGF-β1 construct and active TGF-β1 was expressed by

the AdTGF-$\beta1^{223/225}$ construct. TGF-β1 was expressed as the spontaneously active protein by using a mutant in which Cys223 and Cys225 in the TGF-β1 pro-peptide were converted to Ser. This results in the dissociation of the pro-peptide and secretion of bioactive TGF-β1 (23,24,18). The adenoviruses were amplified in 293A cells and titres were estimated from the Tissue Culture Infectious $Dose_{50}$ ($TCID_{50}$), so that the multiplicity of infection (MOI) could be established.

A. Cell Cultures and Gene Transduction

The procedure of Rheinwald and Green (25) was used for cultivation of human foreskin keratinocytes using serum-free keratinocyte medium (KSFM) (GIBCO, Grand Island, NY) supplemented with bovine pituitary extract (25 μg per ml) and epidermal growth factor (0.5 ng per ml). Primary cultured keratinocytes at passages 3-5 were used. To establish the fibroblast cultures normal skin punch biopsies, obtained from patients undergoing elective reconstructive surgery, were established in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, as previously described (26). Strains of dermal fibroblasts at passages 4-6 were used in this study. Keratinocytes were then grown alone in the upper chamber on a permeable Millicell™ culture plate insert (Millipore, Bedford, MA). These inserts have a pore size of 0.4 μm and are coated with 0.3 mg bovine type I collagen. Dermal fibroblasts were grown in the lower chamber of the co-culture system.

Keratinocytes were plated at a density of 4×10^5 cells/insert and incubated 24 hours. An MOI of 0.5 was used to transduce the keratinocytes with the adenoviruses

overnight. The keratinocytes were washed and the inserts were placed into empty 6 well plates or plates containing 1×10^5 fibroblasts/well and 4 ml fresh media (49% KSFM, 49% DMEM, 2% FBS) was added. The cell cultures were then incubated for 48 hours and conditioned medium was collected and tested for the presence of active and latent of TGF-β1.

B. Fibroblast Proliferation

Co-cultures were established as stated above using two strains of keratinocytes each in combination with two strains of fibroblasts (four separate experiments). Keratinocytes were transduced with either LacZ, TGF-β1 or AdTGF-$\beta1^{223/225}$ prior to co-culturing with fibroblasts. Each condition was tested in triplicate. Following a 48 hour incubation, fibroblasts were counted using a Coulter counter (Model ZM, Coulter Electronics, Bedfordshire, England).

C. ELISA for TGF-β1

To determine the concentration of TGF-β1, a sandwich ELISA was used based on the procedure reported by Danielpour et al. (27). Briefly, 96-well plates were coated with 100 μL per well of mouse monoclonal antibody to human TGF-β (R&D Systems, Mineapolis, MN) at a concentration of 1 μg/mL in phosphate-buffered saline (PBS). The plates were incubated for 3 h at room temperature (RT) followed by 16 h at 4°C. After washing twice with PBS-Tween-20 (PBS-T), the plates were blocked with 1% bovine serum albumin (BSA, crystallized, Sigma) for 60 min at RT and washed twice with PBS-T. To activate TGF-β1, the culture medium was acidified with 12 uL/500 μL conditioned

medium of 3N HCl for 15 min at RT and neutralized with 35 μL of 1M HEPES/ 5 N NaOH (5:2). One hundred microliters of the acidified/neutralized samples was added to each well and the plates were then incubated at 37°C for 1 h. After washing with PBS-T, the plates were incubated with 100μL per well of chicken anti-human TGF-β (R&D Systems) at a concentration of 2.5 μg/mL for 1 h. After washing five times with PBS-T, the plates were incubated with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) at RT for 1 h followed by washing 5 times with PBS-T. After addition of the substrate (o-nitrophenyl phosphate, 1.5 mg/mL, Sigma), the plates were incubated at RT for 1 h and the optical density was read using a THERMOmax (Molecular Devices, Menlo Park, CA) microplate reader at a wavelength of 405 nm. Serial dilutions (0, 125, 250, 625, 1 250, to 2500 pg/mL) of recombinant human TGF-β1 (R&D Systems) were used to prepare a standard curve.

D. TGF-β1 Timecourse

Keratinocytes were plated at 1.5 x 10^6 cells per 75 cm^2 flask. Cells were transduced with an MOI of 0.5 for each adenovirus or incubated in medium alone. Conditioned medium was collected every two days and replaced with new medium. The TGF-β1 ELISA was performed for each timepoint. Total TGF-β1 was measured following acidification and active TGF-β1 was evaluated by measuring non-acidified samples.

E. RNA Extraction and Hybridization

Following 48 hour incubation, culture medium was removed and cell layers were lysed in 2 mL of guanidinium thiocyanate (GITC) as previously described (28). Total

RNA was extracted by the procedure of Chirgwin et al. (29), 10 μg of total mRNA from each treatment was separated by electrophoresis, and blotted onto nitrocellulose filters. Filters were then baked under vacuum for 2 h at 80°C and prehybridized in a solution containing 50% formamide, 0.3 M sodium chloride, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1x Denhardt's solution (1x = 0.02% BSA, Ficoll, and polyvinylpyrrolidone), 0.005% salmon sperm DNA, and 0.005% poly (A) for 3-4 h at 45°C. Hybridization was performed in the same solution at 45°C for 16-20 h using cDNA probes for either human TGF-β1, type I collagen, collagenase, or 18S ribosomal RNA. The probe for 18S rRNA was used to ensure equal loading of samples. The probes were labeled with ^{32}P-α-CTP (DuPont Canada, Streetsville, Mississauga, Ontario, Canada) by nick translation. Filters were initially washed at RT with 2x SSC (1x = 0.15 M sodium chloride, 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) for 30 min, then for 20 min at 65°C in 0.1x SSC and 0.1% SDS solution. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose filters at -70°C in the presence of an enhancing screen. The cDNA probes for 18S ribosomal RNA and collagenase were obtained from the American Type Culture Collection (Rockville, MD). The other cDNA probes were gifts: TGF-β (Dr. G.I. Bell, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology and Medicine, University of Chicago, IL) and type I procollagen (Drs. G. Tromp, H. Kuivaniemi and L Ala-Kokko, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA).

F. Luciferase Assay for TGF-β1

Conditioned medium was collected after 48 hours and assayed for TGF-β1 bioactivity using mink lung cells bearing the plasminogen activator inhibitor-1 promoter-luciferase (PAI/L) construct (22). Mink lung epithelial cells (MLEC) tansfected with this construct (a generous gift from Dr. Daniel Rifkin, New York University Medical Center, New York, NY) were cultured in DMEM supplemented with 10% FBS, L-glutamine (2mM), penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL), and amphotericin B (250 ng/mL). Samples were acid activated (pH 3) for 15 min followed by neutralization. Conditioned medium collected from TGF-β1 and TGF-$\beta1^{223/225}$ transduced cells was also analyzed without acid activation to determine the relative amount of spontaneously active TGF-β1. Samples known to contain high concentrations of TGF-β1 were diluted in 0.1% pyrogen-poor BSA (w/v) (Pierce, Rockford, IL) in serum-free DMEM. A standard curve was constructed (0-500 pg/mL) using recombinant human TGF-β1 (R&D Systems) diluted in 0.1% BSA in DMEM.

Transfected MLECs were plated at a density of 1.6×10^4 cells/well in 96-well tissue culture plates. The cells were allowed to attach for 3 h at 37°C in an atmosphere of 5%CO_2. The medium was replaced with conditioned media or standards and incubated for 14 hours at 37 °C. Cells were then washed with PBS and lysed using 50 μL of 1× cell lysis buffer (Analytical Luminescence, San Diego, CA) for 20 min at room temperature on a rotating platform. The cell lysates (45 μL) were then transferred to clear polypropylene scintillation vials into which 100 μL of both substrate A and B (Analytical

Luminescence) were added and samples were immediately analyzed using the Beckman LS 6000 TA scintillation counter.

G. Statistical Analysis

For ELISA and PAI/L data, the differences between the LacZ transduced control cells and either AdTGF-β1 or AdTGF-$\beta1^{223/225}$ transduced cells were evaluated using a two-tailed non-parametric test (Alternate Welch t test). Samples were measured in triplicate. Data are presented as means ± 1 standard deviation. Student t-test was used for statistical analysis of mRNA results. P-values <0.05 were considered significant for all statistical tests.

V. RESULTS

The kinetics of TGF-β1 expression were studied over 12 days. Conditioned medium was collected every 48 hours for ELISA measurement of active and total TGF-β1. There were significantly higher levels of TGF-β1 released from keratinocytes transduced with AdTGF-β1 or AdTGF-$\beta1^{223/225}$ compared to the LacZ control at all times for at least 12 days (Fig 2-1, Panel A). The level of constitutively active TGF-β1 released from AdTGF-$\beta1^{223/225}$ in non-acidified samples was also significantly higher than with AdTGF-β1 at all times (Fig 2-1, Panel B).

To determine whether TGF-β1 produced by the transduced keratinocytes was able to pass through the insert membrane to influence dermal fibroblasts grown in the lower chamber, medium was collected from the top and bottom chambers and analyzed

following acid activation using the TGF-β1 ELISA (data not shown). There was no significant difference in the concentration of TGF-β1 in the upper and lower chambers. This finding shows that diffusion of TGF-β1 is not impeded by the insert.

TGF-β1 released from keratinocytes was shown to increase fibroblast proliferation. Keratinocytes transduced with either AdTGF-β1 or AdTGF-$\beta1^{223/225}$ significantly increased the proliferation of fibroblasts in the lower chamber compared to the LacZ control ($P<0.0001$) (Fig 2-2).

To demonstrate that cells transduced with the AdTGF-$\beta1^{223/225}$ construct produced enhanced levels of active TGF-β1, whereas those transduced with the AdTGF-β1 construct did not, active TGF-β1 was measured by ELISA in samples which were not acidified (Fig 2-3). The conditioned medium derived from keratinocytes transduced with AdTGF-$\beta1^{223/225}$ had high mean levels of active TGF-β1 (731 ± 198 and 818 ± 265 pg/mL) for keratinocytes alone and keratinocytes co-cultured with fibroblasts respectively, compared to keratinocytes transduced with AdTGF-β1 (31 ± 29 and 36 ± 39 pg/mL) alone and with fibroblasts, respectively. The difference between the level of active TGF-β1 from cells transduced with AdTGF-$\beta1^{223/225}$ and those transduced with AdTGF-β1 or was found to be extremely significant ($P<0.0001$).

To examine whether the level of protein production is consistent with TGF-β1 gene expression, TGF-β1 mRNA was analyzed using Northern analysis (Fig 2-4). Doublet bands were seen in lanes containing keratinocytes transduced with either

AdTGF-β1 and AdTGF-β1$^{223/225}$. An increase in endogenous TGF-β1 was also noted in fibroblasts which were co-cultured with the genetically modified keratinocytes.

The size of the mRNA transcript is smaller for porcine AdTGF-β1 and AdTGF-β1$^{223/225}$ compared to endogenous human TGF-β1. The autoradiograph shows that there are two transcripts present in keratinocytes which have been transduced with either AdTGF-β1 (appears as a doublet) or AdTGF-β1$^{223/225}$, whereas only one transcript was seen in the controls and in fibroblast (endogenous TGF-β1 mRNA). This indicates that the keratinocytes were successfully transduced with the porcine cDNA TGF-β1 constructs and that the fibroblasts in co-culture with the transduced keratinocytes were not subsequently infected by adenovirus. Each blot was probed with a cDNA specific for 18S rRNA as a loading control.

To compare the levels of total TGF-β1 released by the keratinocytes transduced by the latent and active constructs, samples were acidified and total TGF-β1 was measured in medium from keratinocytes alone (Fig 2-5, Panel A) and those co-cultured with dermal fibroblasts (Fig 2-5, Panel B).

The concentration of total TGF-β1 in conditioned medium from AdTGF-β1 and AdTGF-β1$^{223/225}$ transduced keratinocytes both alone and co-cultured with fibroblasts was significantly higher than that from the AdLacZ transduced keratinocyte control ($P<0.0001$). Keratinocytes alone transduced with AdTGF-β1 and co-cultured with

fibroblasts exhibited significantly higher levels of total TGF-β1 compared to those transduced with AdTGF-$\beta1^{223/225}$.

In order to examine whether the TGF-β1 released from transduced keratinocytes was biologically active, the PAI/L assay was performed. Following acid activation, the relative concentrations of total TGF-β1 followed the same trends as those seen by ELISA, for both keratinocytes alone (Fig 2-6, Panel A) and in co-cultures of keratinocytes with fibroblasts (Fig 2-6, Panel B).

In general, the level of total TGF-β1 released from latent TGF-β1 transduced cells was markedly higher than from AdTGF-$\beta1^{223/225}$ transduced keratinocytes. However, as shown in Figure 2-7, without acidification, the biological activity of AdTGF-$\beta1^{223/225}$ transduced keratinocytes was significantly higher than both the LacZ control ($P<0.0001$) and AdTGF-β1 ($P<0.0001$).

To demonstrate whether TGF-β1 could affect the fibroblasts in the lower chamber, total RNA was extracted and analyzed by Northern analysis. The results show that fibroblasts cultured with latent TGF-β1 transduced keratinocytes exhibited a significant increase in type I collagen mRNA ($P<0.05$) (Fig 2-8A and B) compared to the LacZ control. However, the expression of collagenase mRNA was not significantly altered in response to latent TGF-β1 (Fig 2-9A and B). On the other hand, fibroblasts co-cultured with keratinocytes transduced with the active TGF-β1 construct had significantly higher levels of type I collagen mRNA ($P<0.005$) (Fig 2-8A and B) as well as

significantly lower levels of collagenase mRNA ($P<0.05$) (Fig 2-9A and B) compared to the LacZ control.

To quantify the expression of collagenase and type I collagen mRNA, the intensity of signals were evaluated by densitometry using 18 S rRNA as a loading control. The level of mRNA between experiments was normalized using the LacZ control and results for each experimental group were expressed as a percentage of the LacZ control.

VI. DISCUSSION

The purpose of this study was to establish a keratinocyte/fibroblast co-culture system in which keratinocytes release either active or latent TGF-β1. In this system, the effects of latent and active TGF-β1 on underlying fibroblasts can be examined. Replication deficient adenoviruses were used to transduce human keratinocytes. Adenovirus was chosen because of its capacity to transduce both replicating and non-replicating cells and consequent high efficiency of gene transfer. Unlike retroviruses, which may cause insertional mutagenesis, adenoviral replication occurs episomally, that is outside the host cell nucleus. Gene therapy with adenoviruses may be preferred to recombinant TGF-β1 protein therapy due to the relatively high expense and short half-life of the protein (30,31). Keratinocytes turn over rapidly and if an undesirable outcome due to transduction occurs *in vivo*, the epidermis can be removed (32). Also, adenoviruses do not integrate into the host cell genome and are eventually lost through cell replication.

The transient nature of this system is preferred as the gene product will be expressed during wound healing and will be undetectable as wound closure is completed.

Numerous studies have used TGF-β1 in the context of wound healing. TGF-β1 has been applied as a recombinant protein (33,34), naked cDNA (35), in viral vectors (36,18), and expressed by transgenic mice (17,37,38). The system used for delivery of TGF-β1 will have a significant effect on the level and duration of protein expression. This in turn will determine the extent of the influence on formation of extracellular matrix during wound healing.

The concentration of TGF-β1 required to stimulate collagen and elastin production *in vitro* has been established as 10 ng/mL (39). It is expected that *in vivo* at least ten times this concentration will be required to get a similar rise in ECM proteins due to the potential lack of bioavailability of TGF-β1 in this complex system. This very high concentration of growth factor would be very difficult to sustain over a period of days or perhaps weeks until the wound is healed. However, an adenoviral vector might enable high levels of protein to be expressed and the appropriate concentrations could be determined by adjusting the infectious dose of the virus.

We have demonstrated that the adenoviruses are capable of transducing the human keratinocytes without infecting the underlying dermal fibroblasts. We were able to show that both the latent and active forms of TGF-β1 were produced and were biologically active. The TGF-β1 released by keratinocytes was able to traverse the

membrane insert in the co-culture system and affect the underlying fibroblasts. Interestingly, in addition to changes in fibroblast collagen and collagenase mRNAs, we also noted autoinduction of TGF-β1 mRNA in fibroblasts co-cultured with the genetically modified keratinocytes (Fig 2-4). Others have shown that TGF-β1 can induce its own positive feedback loop (40-43).

The effects of normal keratinocytes on dermal fibroblasts have been reported. Keratinocytes and keratinocyte conditioned media have been shown to increase fibroblast proliferation while decreasing collagen synthesis. It has been postulated that keratinocytes may either decrease fibroblast production of collagen, increase production of collagenase or another metalloproteinase, or that keratinocytes may decrease the production of a proteinase inhibitor (44).

TGF-β1 has been shown to increase collagen synthesis (33,36) by dermal fibroblasts in culture. However, it is not clear whether TGF-β1 can increase the synthesis of ECM proteins in the presence of other cytokines and growth factors released by keratinocytes. Here we demonstrate that culturing fibroblasts with keratinocytes reduces the level of type I collagen mRNA compared to fibroblasts alone, and that the levels can be restored by TGF-β1. We have also observed significant changes in collagenase mRNA with active TGF-β1. Fibroblasts alone express very little collagenase mRNA but after coculture with keratinocytes, collagenase mRNA is greatly upregulated. With active TGF-β1 treatment, levels are significantly decreased although not to the level seen in fibroblasts alone. This may be due to the influence of keratinocyte derived cytokines

which TGF-β1 alone may not be able to completely abrogate. Edwards *et al.* (3) had shown that TGF-β1 was capable of reducing the induction of collagenase expression by EGF or bFGF to control levels, where the effect was most pronounced in the presence of other growth factors as opposed to TGF-β1 alone.

The extent to which TGFβ-1 is able to elicit an effect on the wound depends on its activation. Latent TGF-β1 cannot usually bind to its receptor unless it is first released from the LAP or a conformational change occurs. As many cell types possess TGF-β1 receptors, activation of latent TGF-β1 is an important regulatory step for the function of TGF-β1 *in vivo*. *In vitro,* it has been demonstrated that the close proximity of two different cell types can result in activation (45). Latent TGF-β1 has been shown to bind to M6P/IGF-II receptors on cell surfaces via the phosphorylated glycosylation sites on the LAP and this binding is inhibited by M6P (46). Ghahary *et al* (9) demonstrated that the ability of different cell types to activate latent TGF-β1 varies with the number of M6P/IGF-II receptors. By using ^{125}I-labeled IGF-II, the M6P/IGF-II receptors on fibroblasts and mink lung epithelial cells were quantified. Significantly more were present on fibroblasts and only the fibroblasts and not the Mu1Lu cells responded to latent TGF-β1.

In this study, we performed four separate groups of experiments, each with a different fibroblast cell strain. Each of the cell strains showed an increase in the expression of type I collagen mRNA compared to the LacZ control in response to both active and latent TGF-β1. Although only a very small proportion of latent TGF-β1 was

activated in culture (Fig 2-3), this may have been sufficient to elicit an increase in type I collagen mRNA. The differences in the fibrogenic outcomes of these two growth factor states was apparent in a study of lung fibrosis using both active and latent TGF-β1 (18). The transgene was introduced to the rat lungs using adenoviral vectors, the TGF-β1 protein peaked at day 7 and declined rapidly by day 14. Overexpression of active TGF-β1 and not latent TGF-β1 resulted in prolonged interstitial fibrosis. Active TGF-β1 caused the deposition of collagen, fibronectin and elastin as well as the differentiation of fibroblasts into myofibroblasts. Sime *et al.* (18) did not see an increase in hydroxyproline synthesis with the latent construct, which may be due to the increased complexity of their *in vivo* system compared to our *in vitro* culture system and also to the different cell types involved. The latent TGF-β1 which was activated *in vivo* may diffuse from the wound area, be degraded by proteinases or interact with TGF-β1 binding proteins such as α2-macroglobulin and be cleared from the tissue.

Chan *et al.* (38) developed a transgenic mouse model by targeting latent TGF-β1 to the epithelium using a K14 promoter. The study demonstrated a significantly higher level of type I collagen mRNA in both homozygous and heterozygous animals versus normal controls at day 9 following a full thickness punch biopsy wound. This difference coincided with significantly higher levels of total TGF-β1 as measured by the PAI/L assay. One of the most obvious differences in healing in the transgenic animals was the delay in reepithelialization compared to normal animals which may be due to TGF-β1 directly inhibiting keratinocyte proliferation (46). Reepithelialization may also be affected as a result of TGF-β1 diminishing keratinocyte migration by decreasing the

expression of collagenase. Keratinocytes rely upon the release of metalloproteinases in order to break down ECM so that they may migrate across granulation tissue and dissect eschar from viable tissue (47).

In summary, the model we used allows for a direct comparison of the effects of latent and active TGF-β1 on dermal fibroblasts co-cultured with genetically modified keratinocytes. As the wound environment contains elements required for the activation of latent TGF-β1, the use of latent TGF-β1 genetically modified keratinocytes in the form of either sheets of keratinocytes or a keratinocytes/fibroblast skin substitute should be advantageous over the use of active TGF-β1. In this way, the activation of TGF-β1 is limited to the wound area and the risk of development of fibrosis in other organs due to possible leakage of latent TGF-β1 is minimal.

VII. FIGURES AND LEGENDS

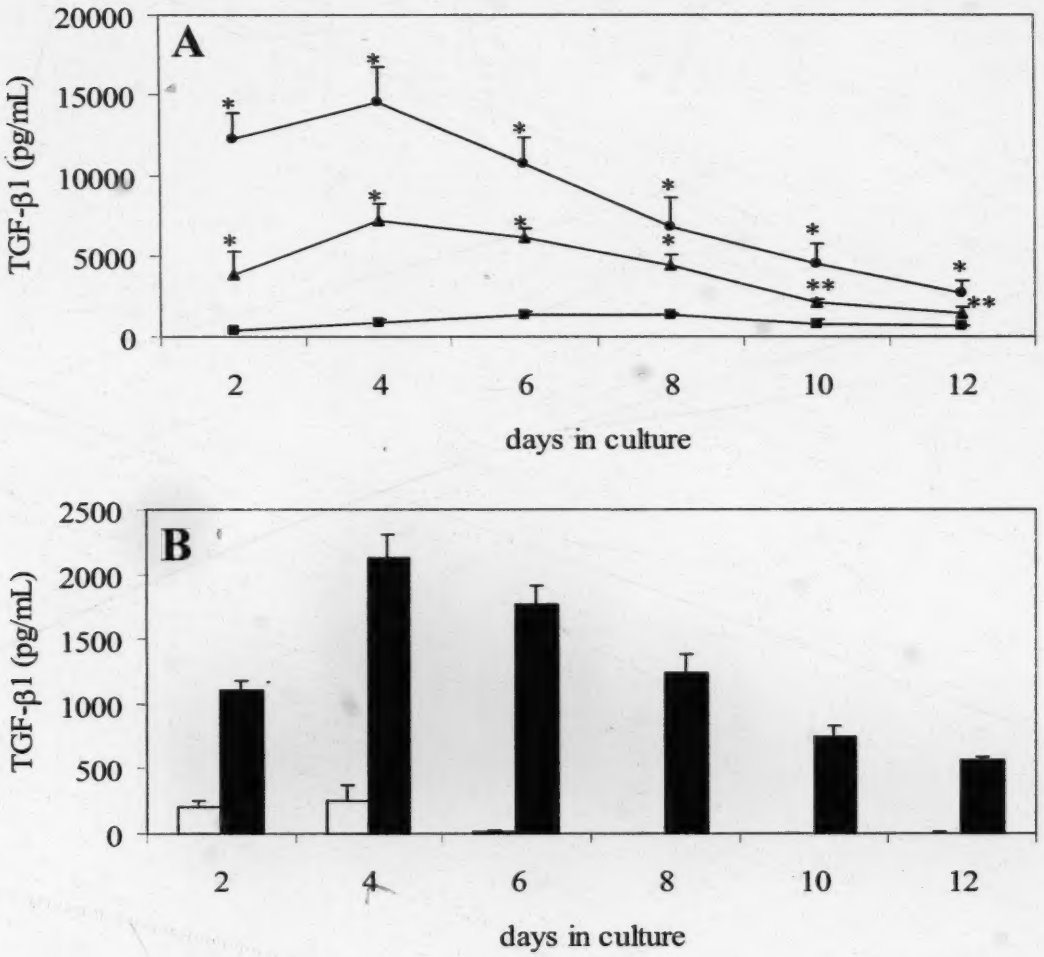


Figure 2-1

Timecourse of release of TGF-β1 from genetically modified keratinocytes. Panel A - Conditioned medium was collected every 2 days and total TGF-β1 was measured using an ELISA following acidification. Compared to the LacZ control (squares), significantly more TGF-β1 released from AdTGF-β1 (circles) and AdTGF-$\beta1^{223/225}$ (triangles) ($^{*}P<0.0001$, $^{**}P<0.001$) . **Panel B -** Constitutively active TGF-β1 was measured in non-acidified samples. Significantly more active TGF-β1 was released from AdTGF-$\beta1^{223/225}$ (black bar) than AdTGF-β1 (open bar) at all times ($P<0.0001$, n=3).

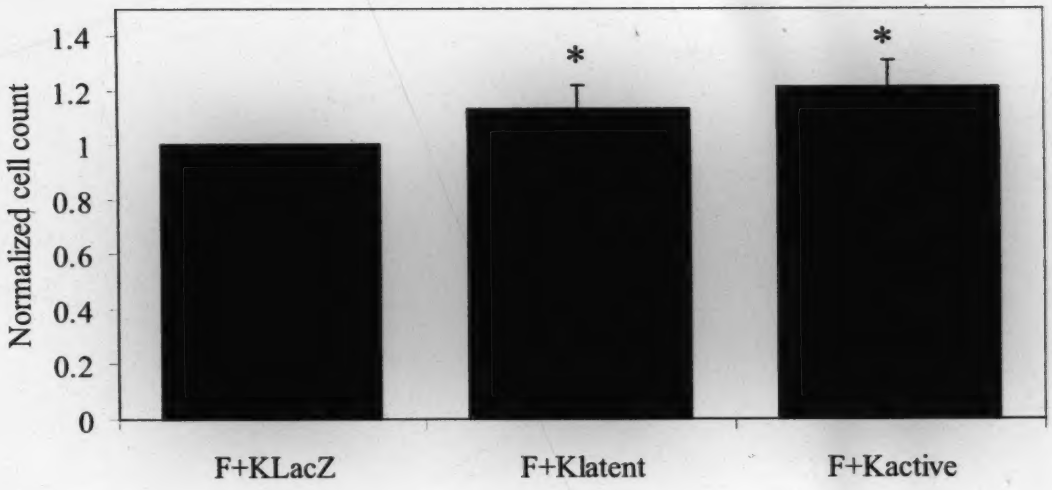


Figure 2-2

TGF-β1 released from genetically modified keratinocytes increases the proliferation of dermal fibroblasts. Four separate experiments were performed with different cell strains. LacZ was normalized (LacZ=1) for all experiments. There was a significant increase in fibroblast proliferation in the presence of AdTGF-β1 or AdTGF-$\beta1^{223/225}$ compared to the LacZ control ($P<0.0001$).

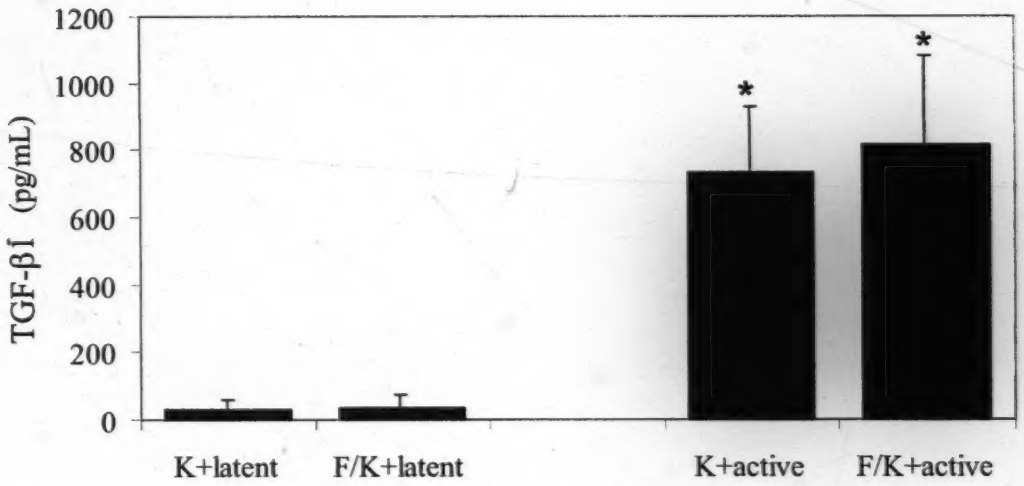


Figure 2-3

TGF-β1 released from keratinocytes transduced with AdTGF-β1$^{223/225}$ was constitutively active. Active TGF-β1 was measured in conditioned medium, which had not been acid activated. Significantly more TGF-β1 was released from keratinocytes transduced with the active construct (AdTGF-β1$^{223/225}$) than from keratinocytes transduced with the latent construct (AdTGF-β1) (* $p<0.0001$). This difference was observed for both keratinocytes cultured alone and co-cultured with dermal fibroblasts (four separate experiments).

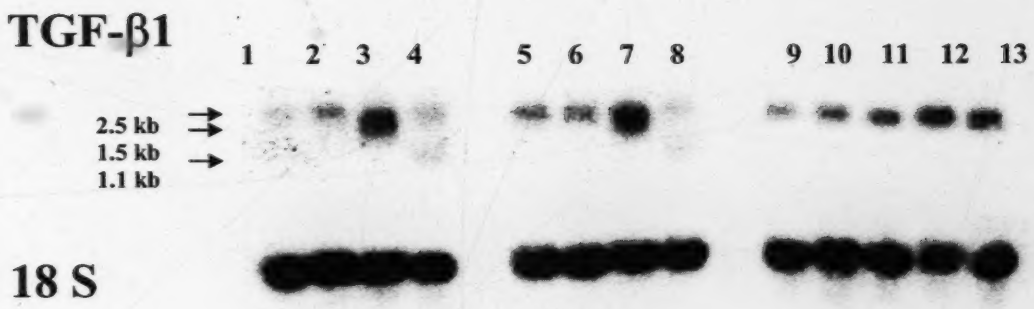
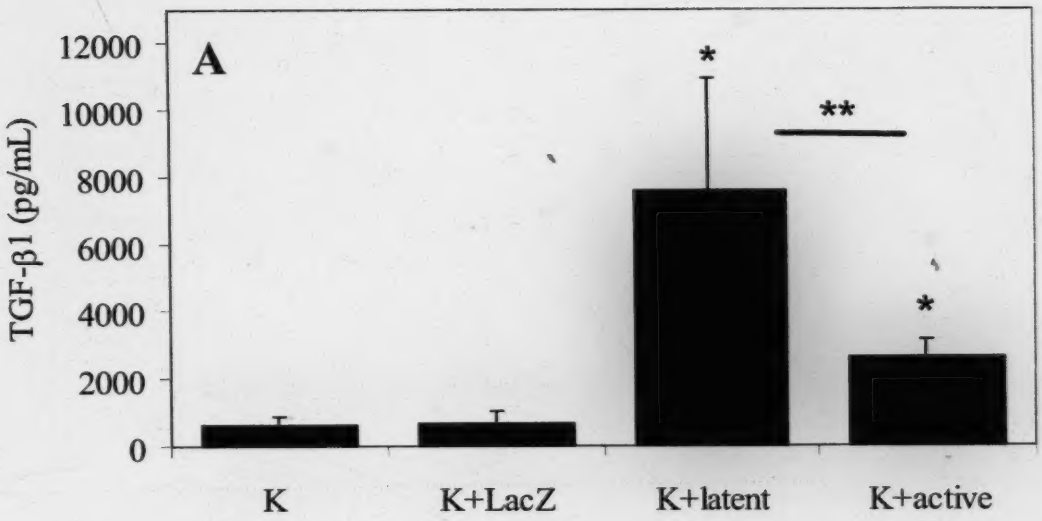


Figure 2-4

Keratinocytes transduced with either AdTGF-β1 or AdTGF-β1$^{223/225}$ express a smaller TGF-β1 mRNA transcript compared to endogenous TGF-β1 mRNA. Representative Northern analysis for TGF-β1 mRNA. Keratinocytes cultured alone, lanes 1-4, keratinocyte mRNA following co-culture with fibroblasts, lanes 5-8, and fibroblast mRNA cultured alone and following co-culture with keratinocytes, lanes 9-13. 18S rRNA was used as a loading control. The TGF-β1 mRNA resulting from the adenoviral constructs is smaller than native mRNA. Two bands were observed in keratinocytes transduced with AdTGF-β1 (lanes 3 and 7) and AdTGF-β1$^{223/225}$ (lanes 4 and 8). Untreated keratinocytes (lanes 1 and 5), keratinocytes transduced with the LacZ control (lanes 2 and 6) and fibroblasts under all conditions (lanes 9-13; F, F/K, F/K+LacZ, F/K+latent, F/K+active) showed only one TGF-β1 mRNA band.


A
TGF-β1 (pg/mL)
12000
10000
8000
6000
4000
2000
0
*
**
*
K
K+LacZ
K+latent
K+active

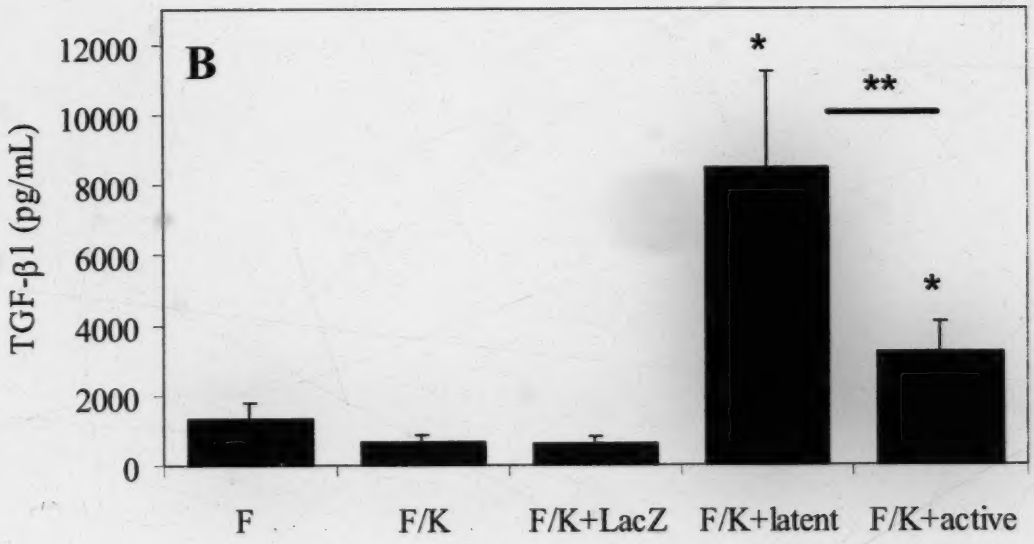
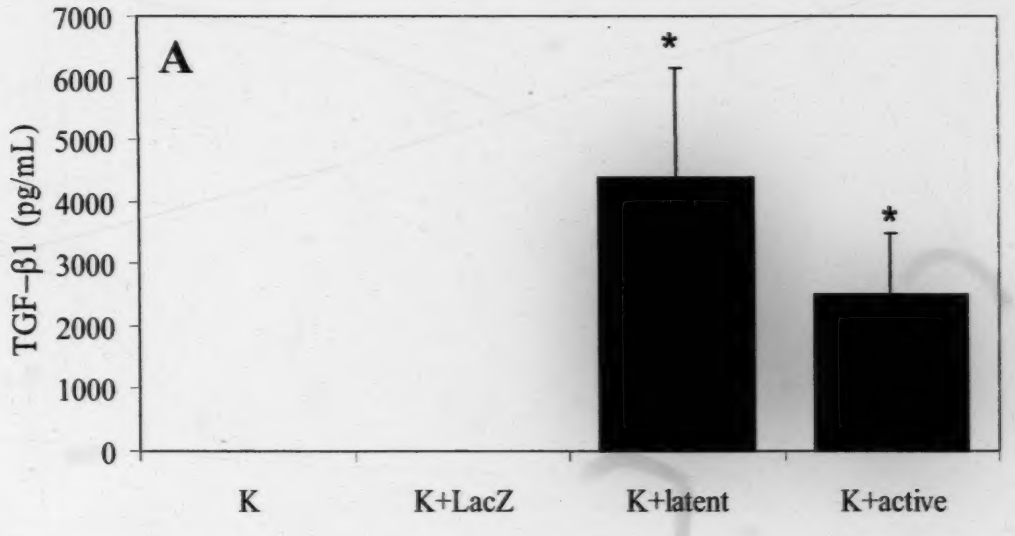

B
TGF-β1 (pg/mL)
12000
10000
8000
6000
4000
2000
0
*
**
*
F
F/K
F/K+LacZ
F/K+latent
F/K+active

Figure 2-5

Total TGF-β in conditioned medium following acidification. Panel A – keratinocytes cultured alone were either untreated (K), or transduced with LacZ, AdTGF-β1 or AdTGF-$\beta1^{223/225}$. **Panel B** – fibroblasts alone and co-cultured with keratinocytes which were either untreated (F), or transduced with LacZ, AdTGF-β1 or AdTGF-$\beta1^{223/225}$. Keratinocytes transduced with either AdTGF-β1 and AdTGF-$\beta1^{223/225}$ had significantly higher levels of TGF-β1 compared to the LacZ control (* $p< 0.0001$). Keratinocytes transduced with AdTGF-β1 had significantly higher levels of total TGF-β1 compared to AdTGF-$\beta1^{223/225}$ ($^{\wedge}p< 0.0001$). (four separate experiments).


A
TGF-β1 (pg/mL)
7000
6000
5000
4000
3000
2000
1000
0
*
*
K
K+LacZ
K+latent
K+active

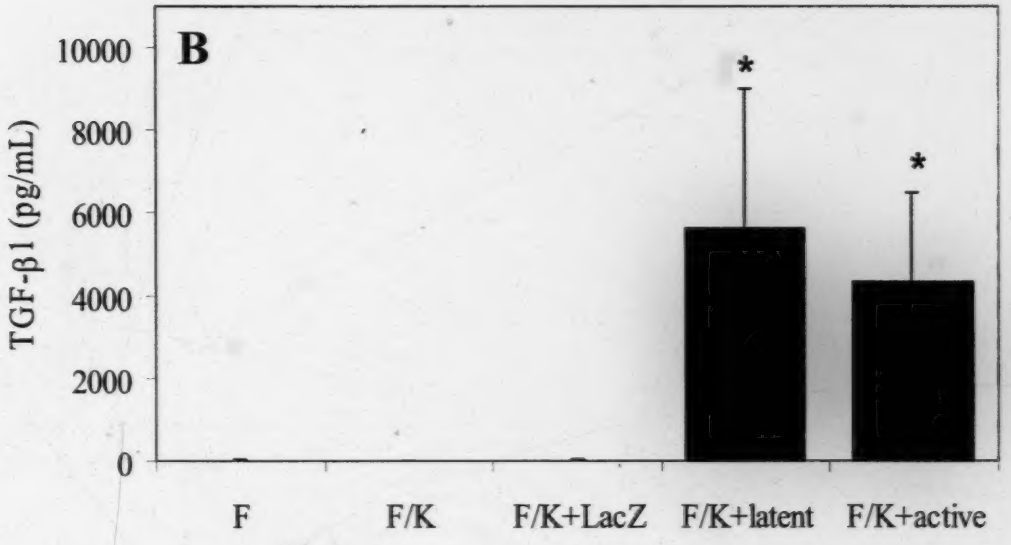

B
TGF-β1 (pg/mL)
10000
8000
6000
4000
2000
0
*
*
F
F/K
F/K+LacZ
F/K+latent
F/K+active

Figure 2-6

The biological activity of total TGF-β1 was measured using the PAI/L assay following acidification of conditioned medium. Panel A – keratinocytes cultured alone (K, transduced with either LacZ, AdTGF-β1 or AdTGF-$\beta1^{223/225}$). **Panel B** – fibroblasts alone or co-cultured with keratinocytes. Keratinocytes transduced with both AdTGF-β1 and AdTGF-$\beta1^{223/225}$ had significantly higher levels of biologically active TGF-β1 compared to the LacZ control (* $p<0.0001$) (2 separate experiments).

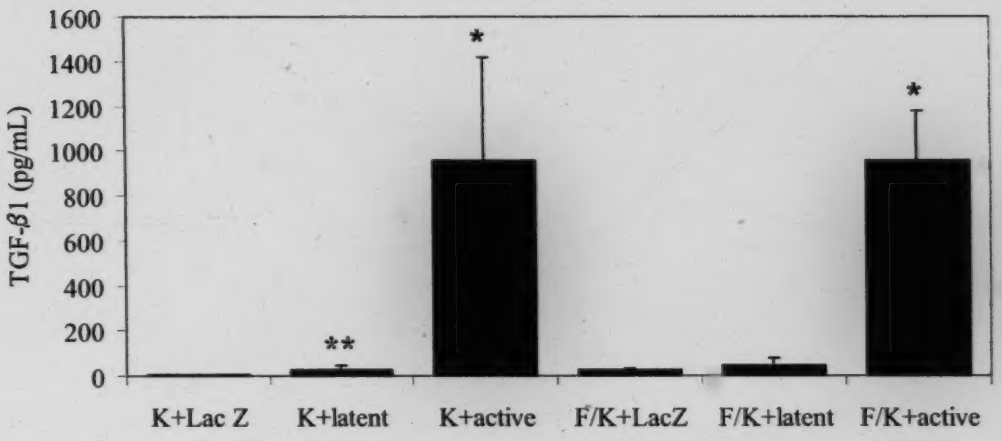
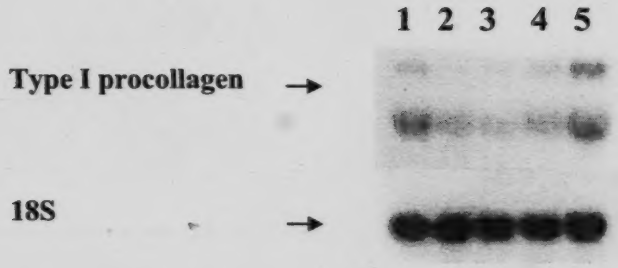


Figure 2-7

The biological activity of active TGF-β1 was measured using the PAI/L assay without acid activation. Keratinocytes transduced with AdTGF-$\beta1^{223/225}$ and cultured alone and in co-culture with fibroblasts released significantly higher levels of functional TGF-β1 than the LacZ controls (* $p<0.0001$). Keratinocytes transduced with AdTGF-β1 and cultured alone also had a significantly higher level of functional TGF-β1 than keratinocytes transduced with LacZ (** $p<0.001$) (2 separate experiments).

A


1 2 3 4 5
Type I procollagen
18S

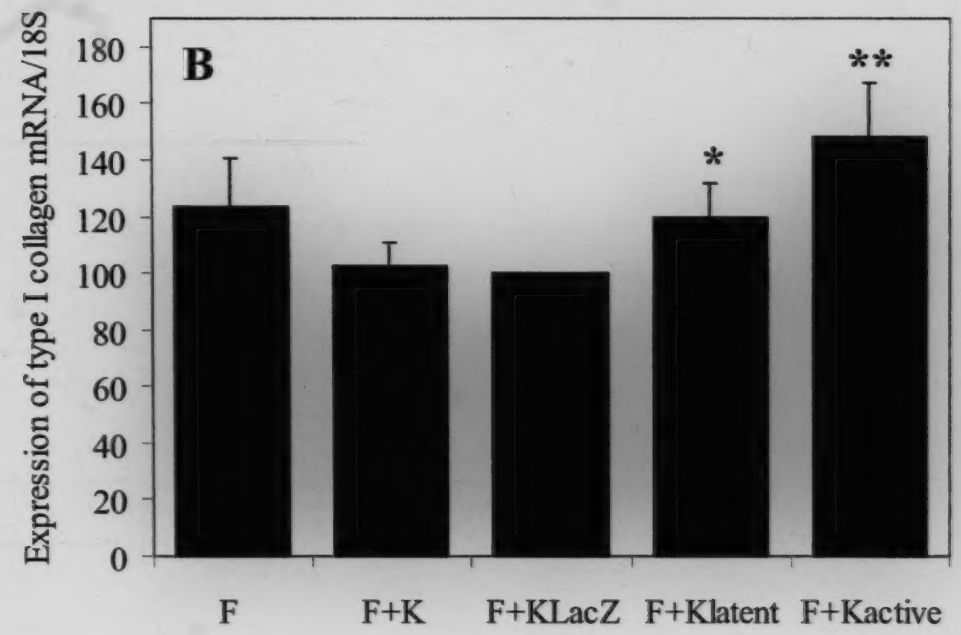
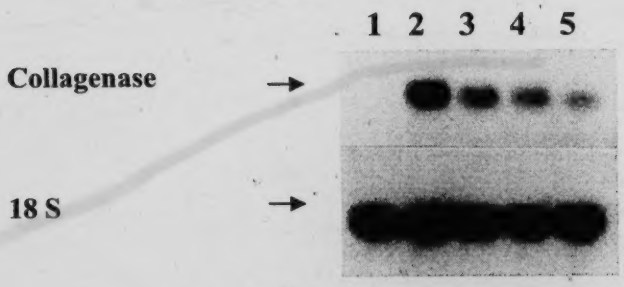

B
Expression of type I collagen mRNA/18S
180
160
140
120
100
80
60
40
20
0
*
**
F
F+K
F+KLacZ
F+Klatent
F+Kactive

Figure 2-8

Expression of Type I collagen mRNA by dermal fibroblasts co-cultured with genetically modified keratinocytes. Panel A. Total RNA was extracted and probed for type I procollagen mRNA by Northern analysis. The autoradiogram shown is representative of four separate experiments, demonstrating the pattern of 5.8 kb and 4.8 kb transcripts (top) corresponding to pro α (I) procollagen mRNA. The bottom autoradiogram shows the profile of the 18S ribosomal RNA used as a control for loading, obtained by rehybridization of the same blot. **Panel B.** Densitometry was performed and the relative intensity of pro α (I) procollagen mRNA to 18S rRNA was calculated, the graph shows combined data from four experiments. Results for each treatment were expressed as a percentage of the LacZ control (lane 3). Lanes 1 and 2 in panel A correspond to F and F/K. Fibroblasts exposed to AdTGF-β1(lane 4) and AdTGF-$\beta1^{223/225}$ (lane 5) had significantly higher levels of pro α (I) procollagen compared to the LacZ control ($P<0.05^{*}$ and $P<0.005^{**}$ respectively, n=4).

A


1 2 3 4 5
Collagenase
18 S

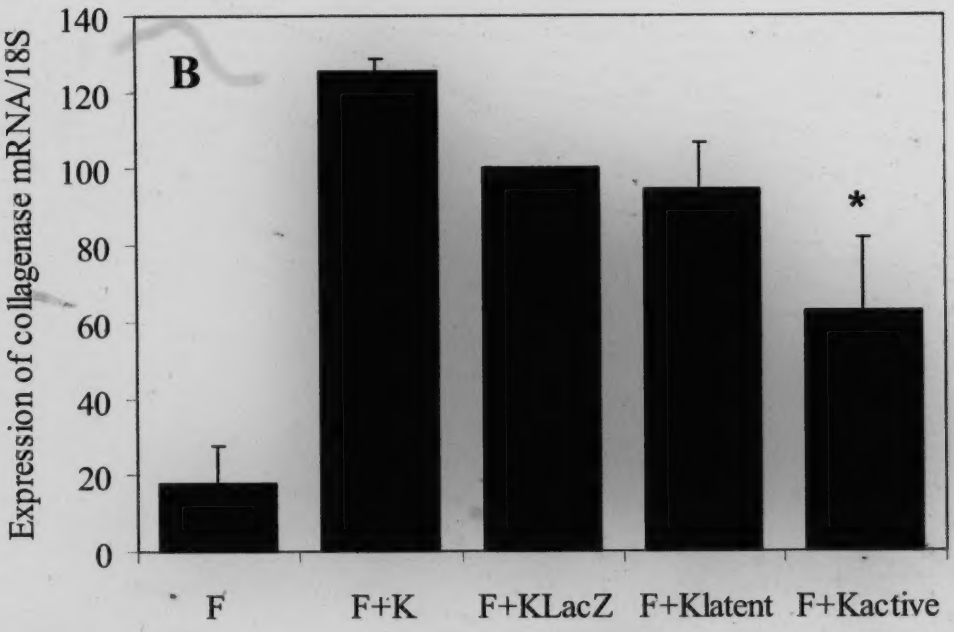

B
Expression of collagenase mRNA/18S
140
120
100
80
60
40
20
0
*
F
F+K
F+KLacZ
F+Klatent
F+Kactive

Figure 2-9

Expression of collagenase mRNA by dermal fibroblasts co-cultured with genetically modified keratinocytes. Panel A. Total RNA was extracted and probed for collagenase mRNA by Northern analysis. The autoradiogram shown is representative of three separate experiments, demonstrating the 2.1 kb transcript (top) corresponding to collagenase mRNA. The bottom autoradiogram shows the profile of the 18S ribosomal RNA used as a control for loading, obtained by rehybridization of the same blot. **Panel B.** Densitometry was performed and the relative intensity of pro α (I) procollagen mRNA to 18S rRNA was calculated. The graph shows combined data from three experiments, with the results of each treatment expressed as a percentage of the LacZ control (lane 3). Lanes 1 and 2 correspond to F and F/K. Fibroblasts exposed to AdTGF-β1(lane 4) did not have significantly lower levels of collagenase mRNA compared to the control, whereas those exposed to AdTGF-$\beta 1^{223/225}$ (lane 5) did. ($P<0.05^{*}$).

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Chapter 3

Obesity, Adipokines and Wound Healing

I. RATIONALE

In the previous study we successfully transduced human keratinocytes with adenoviral vectors bearing the constructs for either latent or active TGF-β1. We then attempted to inject these constructs into the skin surrounding excisional wounds on the JCR:LA-cp rat, a novel model for chronic wound healing. This obese rat model exhibits many of the characteristics of early insulin resistance in humans. While this model has been used extensively in the study of atherosclerosis, it has not yet been studied in the context of wound healing.

Unfortunately we were unable to successfully transduce the surrounding cells of the wounds with the adenovirus. Adenovirus invades target cells by first binding to the Coxsackievirus and adenovirus receptor (CAR) on the cell membrane. CAR expression is low or absent in human fibroblasts (1). Consequently these cells are difficult to infect with Ad5-based vectors (2). The adenoviral vectors used in our study contained the left end of human adenovirus type 5 genome (3). Our initial study with the recombinant adenoviruses involved transducing human keratinocytes *in vitro* and we were successful in showing the expression of the latent and active TGF-β1 transgenes and some biological effects. However, in our *in vivo* model, due to the relative size of our syringe compared to the epidermis we injected most of the adenoviruses into the dermis. We were unable to show successful transduction in the skin, the main reason being that fibroblasts are the primary cell type of the dermis.

Due to the complexity of gene therapy, we decided to change our focus instead to the study of obesity and insulin resistance in wound healing and the characterization of the JCR:LA-cp rat as a novel healing impaired model. The impact of obesity and insulin resistance on a variety of conditions including wound healing is currently an important area of study due to the increasing number of morbidly obese individuals in our society. ThIS next chapter reviews the impact of obesity and insulin resistance on wound healing with a focus on adipokines (adipose tissue-derived cytokines) and animal models used to study the condition.

II. Obesity and Wound Healing in Humans

In 2000, 19.8% of US adults were classified as being obese (4). Obese individuals are at risk for a number of health problems, including the inability to heal wounds properly (reviewed in 5). In obesity, the workload of the heart is increased to supply oxygenated blood to tissues. Adipose tissue is more at risk of ischemia and necrosis due to decreased vascularity which may lead to pressure ulcers. Oxygen is also required for collagen synthesis and leukocyte and phagocytic activities. Although wound healing occurs under hypoxic conditions, the cellular repair processes are oxygen dependent. Reduced oxygenation leads to the production of oxygen free radicals that impair wound healing by damaging keratinocytes, endothelial cells and collagen metabolism. The lack of oxygen and presence of moisture in skin folds also leads to increased risk of infection (6-9) by providing optimal conditions for the growth of anaerobic bacteria (10) as well as Staphylococcus aureus and Pseudomonas aeruginosa (11).

III. Insulin Resistance

In patients with type 2 diabetes, insulin is less able to promote glucose uptake into muscle and fat and inhibit the production of glucose by the liver. The events leading to insulin resistance may be genetic or acquired (Reviewed in 12). Obesity is the principal cause of insulin resistance and is the major predisposing factor in the development of type 2 diabetes (13). Després et al. found that high fasting insulin levels were an independent predictor of ischemic heart disease in men (14).

In type 2 diabetics, tyrosine (Tyr) phosphorylation of insulin receptor substrate (IRS)-1 and -2 and the association of IRS-1 with phosphatidylinositol-3 kinase (PI3-K) are decreased (fig 3-1 - insulin signal transduction) (15). Insulin and Tyr kinase activity are restored with weight loss and insulin sensitivity is improved. The relationship between obesity, insulin resistance and diabetes is not well understood. Shulman et al. demonstrated that muscle glycogen synthesis is the major pathway for glucose metabolism in normal and diabetic patients, and defective glycogen synthesis is important in the development of insulin resistance (16). Proposed mechanisms for the development of insulin resistance include the Randle hypothesis which states that fatty acids compete with glucose for oxidation in muscle resulting in an increase in intracellular glucose concentration and a decrease in muscle glucose uptake and insulin resistance (figure 3-2a) (17). Alternatively, Shulman hypothesized that increases in plasma fatty acid concentrations induce insulin resistance by first inhibiting glucose transport or phosphorylation activity and that results in reduced glycogen synthesis and glucose

oxidation (figure 3-2b) (18-20). Recently the function of adipose tissue as an endocrine tissue has increased the scope of possibilities. Adipose tissue releases numerous cytokines termed "adipokines" which are thought to influence insulin sensitivity. These include leptin, TNF-α, adiponectin and resistin and the concentrations of each of these adipokines are affected by obesity.

IV. Adipokines

The exact mechanisms by which leptin resistance leads to type 2 diabetes, atherosclerosis and impairment of wound healing are not known. Adipocytes were initially thought to serve only as fat storage, to be released when other sources of energy were low. However, recent interest in this cell type has uncovered an enormous amount of information concerning adipocytes and their relationship with dyslipidemia, obesity and type 2 diabetes (21). Adipose tissue acts as an endocrine organ that releases a relatively newly recognized class of factors called adipokines (or adipocytokines) which includes leptin, TNF-α, resistin and adiponectin (22). Leptin is a hormone that is produced in the adipose tissue and targets the hypothalamus to influence satiety. An absence of leptin protein or a defect in leptin signaling results in obesity. However, the story of leptin is becoming increasingly complex. Leptin has been shown to alter insulin sensitivity as there is crosstalk within the signal transduction pathways and leptin is also involved in the immune system by altering Th1/Th2 responses. Interestingly, leptin has been shown to have growth factor properties in that it acts as a mitogen for several cell types including endothelium and epithelium. Increased adipose tissue mass leads to altered secretion of leptin, TNF-α, adiponectin and resistin. There is little doubt that

these factors, all having different functions, interact with one another to influence the effect of insulin on target tissues. The impact of these factors in the progression of type 2 diabetes is an exciting new field of study and the involvement of these factors as a group in wound healing has yet to be investigated.

A. Leptin

Leptin circulates in mouse and human plasma as a 16 kDa protein and plasma levels of leptin are highly correlated with adipose tissue mass (23). Administration of leptin in mice results in a dose-dependent decrease in body weight which is restricted to adipose tissue, not lean body mass (24). Mice deficient in leptin hormone (*ob/ob* mice) resemble starved animals in that they have decreased body temperature, hyperphagia, decreased immune function and infertility (25), all of which are reversed with leptin administration.

There are five splice variants of the leptin receptor gene (OB-R). Sequencing of the original murine cDNA demonstrated a single membrane-spanning receptor of the class I receptor family (26). The closest relatives of Ob-R are gp130 (27), the G-CSF receptor (28), and the leukemia inhibitory factor receptor (29). Only the splice variant ObRb has been shown to mediate intracellular signaling through the activation of the Jak-STAT pathway (30) (figure 3-3). The binding of leptin causes receptor dimerization and phosphorylation of JAK-2 kinase. JAK-2 has intrinsic tyrosine kinase activity, it autophosphorylates and also phosphorylates the leptin receptor on various tyrosine residues. A class of cytoplasmic transcription factors called signal transducers and

activators of transcription (STAT) proteins (STAT-3, STAT-5, STAT-6) are subsequently recruited and phosphorylated (30). Following phosphorylation, STATs dissociate from the receptor complex and relocate to the nucleus. Different pairings of STATs may result in a diversity of signals. Keratinocyte-specific STAT3 deficiency results in severe wound healing impairment (31). In the epidermis, ObRb is expressed only in the basal layer and in hyperproliferative epithelium at the wound edge, whereas ObRa was observed throughout the epidermis (32).

Although insulin has been shown to stimulate adipocyte leptin synthesis (33), a mechanism whereby insulin inhibits leptin receptor signaling has been described, suggesting that hyperinsulinemia contributes to leptin resistance (34). HEK293 cells were used to study the crosstalk between insulin and leptin as these cells bear the ObRb receptor isoform and PI 3-kinase. JAK-2 and IRS-2 were transiently over-expressed in these cells and pre-incubation of these cells with insulin abolished the JAK-2 phosphorylation observed with leptin treatment alone. SHP-1 is a phosphatase that is associated with JAK-2 and is activated by the insulin receptor, its activation results in decreased phosphorylation of JAK-2.

Leptin may not only act on the hypothalamus to signal a reduction in food consumption, but also to notify the pancreas and act as a negative regulator for insulin release (35). This effect is mediated specifically by the leptin receptors found on the islets (36). Recombinant leptin inhibited insulin release from perfused pancreas preparation from *ob/ob* mice (lacking leptin protein) but not Zucker *fa/fa* rats (highly

impaired leptin receptors). Leptin was also shown to inhibit glucose-stimulated insulin secretion by islets in a dose-dependent manner in *ob/ob* mice but not *db/db* mice (lacking functional leptin receptors). In obesity, there is an acquired leptin resistance (37). In this case, insulin levels are not controlled by the action of leptin on the pancreas, and hyperinsulinemia may result.

Leptin receptors have also been localized to the endothelial cells of the vasculature (38). An ample blood supply surrounding adipose tissue would be necessary for its role as an endocrine tissue. The authors found that treatment of endothelial cells with leptin upregulated tyrosine phosphorylation of ObRb and induced the phosphorylation and DNA-binding activity of STAT3. Endothelial cells exhibited a directional migration response toward leptin and also formed capillary-like tubes in 3-dimensional gels. In vivo, Hydron polymer pellets containing PBS, VEGF or leptin were implanted into the corneas of normal or *fa/fa* Zucker rats and neovascularization was assessed. Normal rats exhibited an angiogenic response toward leptin but *fa/fa* Zucker rats did not, although they did respond to VEGF. The investigators concluded that leptin can act in a paracrine fashion by binding to endothelial cells, causing an angiogenic response. Conversely, full thickness excisional wounds in *ob/ob* mice were treated with leptin (1 ug twice daily topically or 5 ug/g body weight i.p.), and although wound closure and epithelial expression of VEGF was improved, angiogenesis in the granulation tissue was not (39). One would expect an increase in VEGF expression to be associated with improved angiogenesis, however, Lauer et al. demonstrated rapid $VEGF_{164}$ degradation in chronic wound lysates due to increased proteolytic activity (40).

The regulation of the immune system in response to diverse pathogens is intricate and essential for survival. The mechanisms of the immune response are now clearer since the identification of the CD4+ T helper subpopulation (Th1 and Th2). Following stimulation by an antigen presenting cell, naïve CD4+ T cells produce IL-2 and then differentiate to secrete specific cytokine profiles (reviewed in 41). Th1 cells secrete proinflammatory cytokines (i.e. IL-2 and IFN-γ, and lymphotoxin) and are involved in macrophage and monocyte-mediated responses. Th2 cells secrete regulatory cytokines (i.e. IL-4, IL-5, IL-6, IL-9, and IL-10) and are involved in antibody production and mast cell and eosinophil proliferation and function.

Leptin is capable of increasing Th1 while suppressing Th2 cytokine production (42). It has been suggested that suppressed cell-mediated immunity during nutritional deprivation is due to a decrease in leptin expression. Leptin treatment of splenocytes from *db/+* mice showed an increase in proliferation that was not seen in *db/db* mice indicating that the effect of leptin on proliferation is through leptin receptor signaling. T cells also showed an increase in IFN-γ secretion in response to leptin indicating that leptin promotes a Th1-like proinflammatory cytokine profile in CD4+ T cells while inhibiting a Th2 cytokine profile. These results further supported the hypothesis that a decrease in leptin levels during starvation acts as a signal to conserve energy (43) in that the cognate immune response, which requires clonal expansion, is inhibited. The induction of Th1 by leptin has been linked to experimentally induced autoimmune diseases such as insulin-dependent diabetes mellitus (44). Interestingly, female mice and humans are

hyperleptinaemic compared to males and have a higher incidence of autoimmune disease, this may be due to hormones or sex-linked genetic factors (45).

In obesity, when leptin resistance occurs, individuals have an increased susceptibility to infection. In wound healing, it has been demonstrated that a severe injury also results in a decreased Th1 response (46). This T cell imbalance leads to generalized immunosuppression and increased susceptibility to infection and sepsis. It is therefore likely that leptin resistance, combined with a severe injury, would cause substantial immunosuppression and contribute to wound healing deficiencies.

Leptin has also been shown to act as a mitogen for epithelial, endothelial, and pancreatic cells *in vitro* (32,47,48). Frank *et al.* (32) studied the influence of leptin on keratinocytes during reepithelialization in *ob/ob* mice. They demonstrated that leptin improved reepithelialization of excisional wounds and accelerated wound healing in *ob/ob* mice. Leptin-treated animals exhibited a highly organized epithelium; whereas, the PBS-treated control wounds were disorganized and atrophied. The leptin was administered systemically and topically to ensure that systemic leptin was not just influencing the diabetic phenotype resulting in improved healing. In the same study, leptin receptor (ObR) expression was measured. PBS treated *ob/ob* mice demonstrated a delay in ObR expression compared to wild-type mice and leptin-treated *ob/ob* mice indicating that leptin protein can correct the expression of its own receptor. As ObRb receptors are localized to proliferating keratinocytes, leptin may act on these receptors to mediate mitogenesis.

Recently, the presence of leptin has been detected in the wound fluid of pigs (49). The level of leptin in partial thickness wounds was shown to peak within the first 3 days following wounding (4.8-10.2-fold). The presence of leptin during this time supports the hypothesis that leptin plays a role in reepithelialization, as this phase of healing takes place 1-6 days following a partial thickness wound. This is in contrast to Stallmeyer (50) who reported a decrease in leptin expression following wounding. The discrepancy may be due to a difference in animal (pig vs mouse), in wounding (partial thickness vs full thickness) or in the type of leptin assay used (bioassay vs radioimmunoassay). Marikovsky *et al.* (49) propose that the partial thickness wound results in leptin secretion from the subcutaneous fat in the pig while the mouse used by Stallmeyer lacks the necessary adipose tissue.

Wound healing deficiencies have been described in *db/db* mice. The impairment of wound healing in the *db/db* mouse is characterized by a delay in cellular infiltration, granulation tissue formation and reepithelialization, as well as reduced angiogenesis and collagen content (51, 52). *Db/db* mice were found to have low vascular endothelial growth factor (VEGF), delayed healing and decreased tensile strength. A significant reduction in the expression of platelet derived growth factor (PDGF) and PDGF receptor in *db/db* mice has been shown in unwounded skin and following wounding (53). The wound healing impairment seen in these animals can be improved with topical recombinant PDGF-BB (54). Treatment with rPDGF-BB resulted in increased cellular infiltration and capillary ingrowth, giving rise to increased granulation tissue formation.

The expression of insulin-like growth factor (IGF)-I and IGF-II during wound healing in *db/db* mice are delayed compared to normal littermates (55). While levels of IGF-I in the *db/db* mice only reached 50% of the control, IGF-II were increased four fold by day 10. Usually, IGF-II is highly expressed in fetal tissue and declines after birth. However, in diabetic animals IGF-II appears to play an important role. Other studies have shown that IGF-I combined with PDGF did not have an additive effect on healing of *db/db* mice, while IGF-II and PDGF did (56). This indicates different functions of IGF-I and IGF-II during wound healing, and it has been speculated that IGF-II may be compensating for IGF-I which may be sequestered by binding proteins. Also, apoptosis in the *db/db* mouse is delayed and the delay is reversed with IGF-II and PDGF combined therapy (57).

Besides growth factors, derangement of proteolytic enzymes may also lead to delayed healing. The expression of the matrix metalloproteinase (MMP)-2 (72 kd type IV collagenase, gelatinase A) and MMP-9 (92 kd type IV collagenase, gelatinase B) have been studied in *db/db* mice (58). In humans, MMP-2 and MMP-9 are higher in chronic leg wounds and pressure ulcers compared to acute wounds (59). Prior to wounding, the non-diabetic littermates had significantly higher levels of MMP-2 in unwounded tissue than *db/db* mice and neither group had detectable levels of MMP-9. Following wounding, the expression of latent gelatinases peaked earlier in the *db/db* mice and there were more active gelatinases present in their wounds compared to normal littermates.

This model parallels the clinical data insofar as gelatinase expression in chronic wounds leading to excessive proteolytic activity.

B. Tumor Necrosis Factor-alpha

There is evidence that TNF-α contributes to insulin resistance and type 2 diabetes. Increased levels of TNF-α have been measured in cultured white adipose tissue of high fat fed mice and rats (60). Although the levels of TNF-α in the serum of both lean and obese humans is very low, the levels in the adipose tissue and muscle of insulin-resistant and diabetic patients are significantly higher than in insulin-sensitive patients (61-63).

Prolonged exposure to TNF-α has been shown to increase insulin resistance (64), and there are several metabolic effects of TNF-α which lead to insulin resistance. TNF-α can inhibit expression of GLUT4 (glucose transporter 4) and decrease glucose transport (65). A recent study found that insulin resistance in obese patients is positively correlated with triglyceride content within muscle cells and TNF-α gene expression and inversely correlated to GLUT4 expression (66). Second, TNF-α can antagonize synthesis/action of PPARγ (peroxisome proliferator-activator receptor γ). PPARγ is a nuclear receptor involved in adipogenesis and regulation of adipocyte function by increasing fatty acid uptake by adipocytes, thus lowering FFA (free fatty acid) levels. Thiazolidinedione drugs are PPAR-γ agonists and insulin sensitizers. They abrogate the effect of TNF-α on fat cells and may inhibit TNF-α synthesis in fat (67). Third, TNF-α promotes lipolysis and increases FFA levels. Thiazolidinediones prevent TNF-α-mediated lipolysis (68).

And finally, TNF-α may promote serine phosphorylation of IRS-1, thus decreasing insulin-mediated signaling (69).

Chronic wounds are characterized by elevated pro-inflammatory cytokines such as TNF-α, IL-1β and increased proteases such as matrix metalloproteinases (MMP-2 and MMP-9) and serine proteases (70,71). Chronic wounds have also been shown to contain lower levels of tissue inhibitors of metalloproteinases (TIMPs) (72-74). MMP-9 expression is higher in chronic wounds and it functions in the digestion of basement membrane components such as type IV collagen and the type VII collagen which anchor the basement membrane to the dermis. It exists as an inactive 92-kDa form which can be converted to an 82-kDa active form by pro-MMP-9 activator (pM9A), a tissue-associated chymotrypsin-like proteinase (75). In normal tissue, MMP-9 is expressed in very low quantities while TIMP-1 is constantly expressed. Following injury, TGF-β induces pro-MMP-9 expression which then binds to TIMP-1 and maintains its latency. TNF-α down regulates TIMP-1 and pro-MMP-9 is then activated by pM9A.

Wound fluid from chronic leg ulcers has been shown to have less effect on promoting fibroblast proliferation *in vitro* compared to fluid from healing chronic wound fluid from the same patients (76). The chronic wounds were shown to have increased levels of TNF-α, IL-1, IL-6 but no significant difference in PDGF, EGF, bFGF, or TGF-β compared to healing wounds. It was suggested that non-healing wounds may result from increased inflammation rather than a deficiency of growth factors. Inflammation requires proteolytic enzymes for the destruction and remodeling of tissues. Collagenase breaks

down collagen during inflammation (77) and prostaglandin (PGE_2) induces intracellular proteases (78). Dayer *et al.* (79) demonstrated that TNF-α was able to induce collagenase and PGE_2 production from synovial cells and fibroblasts. TNF-α has also been shown to inhibit collagen and fibronectin synthesis by fibroblasts (80).

C. Adiponectin

Adiponectin is the most abundant protein in adipose tissue (81) and it comprises 0.01% of total plasma protein (82). Very recently, Yamauchi et al. cloned two receptors for adiponectin, AdipoR1 and AdipoR2 (83). The receptors are predicted to contain seven transmembrane domains but are structurally and functionally distinct from G-protein-coupled receptors. The expression of AdipoR1 has been demonstrated in muscle, while AdipoR2 is predominantly expressed in liver. The investigators demonstrated that the receptors were able to mediate increased AMP-activated protein kinase (AMPK) and PPAR-α ligand activity, fatty acid oxidation and glucose uptake by adiponectin. Since then, the expression of both Adipo1 and Adipo2, have also been demonstrated in both human and rat pancreatic β cells (84).

The expression of adiponectin mRNA and protein is reduced in coronary heart disease, and in obese mice and humans (85), which may implicate this protein in the development of insulin resistance. Obese males were found to have up to 53% less plasma adiponectin than lean controls (86). Sex differences in adiponectin plasma concentrations have been observed (87). In one study, men and women with a body mass

index (BMI) >30kg/m^2 were compared. Although the women had a two-fold higher percent body fat, their plasma adiponectin levels were 65% higher. With weight loss, there is an increase in plasma adiponectin and adipose tissue adiponectin mRNA. The expression of adiponectin may be increased during adipogenesis but is prone to negative feedback inhibition in obesity (88). Plasma adiponectin correlates inversely with both adiposity and insulin sensitivity (86, 87). In animal models of insulin resistance and in humans with type 2 diabetes, there is an increase in intramuscular triglycerides; adiponectin may increase the β-oxidation of fatty acids thereby lowering serum triglycerides and free fatty acids (89).

In lipoatrophic mice (mice that lack adipose tissue), and obese mice, adiponectin is reduced (90). Combination treatment using leptin and adiponectin almost completely abolished insulin resistance in the lipoatrophic mice. Treatment of *db/db* mice with adiponectin was shown to improve insulin resistance indicating that adiponectin mediates its effects in a leptin-independent pathway. This study also found that decreased triglycerides with adiponectin treatment were associated with improved insulin signal transduction, as shown by increased insulin-induced tyrosine phosphorylation of the IR and IRS-1.

Adiponectin has a number of other systemic functions, most notably, anti-inflammatory effects on endothelial cells and macrophages. Hypoadiponectinemia has been associated with endothelial dysfunction as decreased adiponectin correlated with resistance vessel endothelial function (91). A significant inverse correlation has been

observed between levels of adiponectin and TNF-α (83, 84). Although adiponectin does not affect TNF-α binding to its receptor, it inhibits TNF-α-stimulated NF-κB activation (92). As leukocyte adhesion to the vascular wall is considered essential for the development of atherosclerosis, the anti-inflammatory effect of adiponectin may protect against atherosclerosis through the decreased expression of adhesion molecules (93), monocyte attachment, and the atherogenic transformation of macrophages to foam cells (94). Conversely, TNF-α has been shown to decrease adiponectin mRNA *in vitro* (86).

The treatment of insulin resistance with adiponectin rather than other anti-diabetic drugs currently used may have numerous advantages (90). Adiponectin may prevent atherogenesis because of its anti-inflammatory properties (92), adiponectin mediates anti-diabetic effects without increasing body weight. Unlike the case with insulin and leptin, and there was no observable adiponectin resistance in obesity and type 2 diabetes. The involvement of adiponectin in wound healing has not been previously studied to our knowledge.

D. Resistin

Resistin (for resistance to insulin) is a protein that has very recently been associated with type 2 diabetes (95). In fasting or diabetic (streptozotocin treated combined with a high carbohydrate diet) animals, resistin expression is low or non-detectable in adipose tissue, but levels rise following feeding or insulin administration (96). The mechanism by which thiazoladinediones affect PPAR-γ was previously unknown, but recently they have been found to decrease the expression of resistin, a new

protein which is only found in adipose tissue (95). Immunohistochemistry showed that resistin was highest in the cytoplasm of adipocytes of white adipose tissue. Resistin is induced during adipocyte differentiation, circulates in mouse serum and is increased in obesity. Immunoneutralization of resistin was found to improve blood glucose and insulin action in mice with diet-induced obesity, whereas administration of resistin did the opposite. Treatment of 3T3-L1 cells with anti-resistin IgG increased insulin-mediated glucose uptake by 42% while purified resistin-F (carboxy-terminal Flag-tag) reduced insulin-stimulated glucose uptake by 37%, indicating that this is a function of resistin. The resistin receptor, signaling pathway and mechanism of resistin action are unknown to date but Steppan suggests that resistin binds to receptors in insulin-responsive tissues and modulates insulin signal transduction (95).

In contrast to the results above, Way et al. (97) determined that in several animal models of obesity including *ob/ob, db/db, tub/tub*, and KKA^{y} mice; resistin mRNA expression is significantly lower in white adipose tissue compared to lean controls. Also, treatment with PPAR-γ agonists increased adipose tissue resistin expression in *ob/ob* mice and Zucker *fa/fa* rats even through there were significant decreases in glucose levels.

The contradictory results of the above studies have not been explained to date. It has been suggested that the differences may be due to methodologies or that resistin does not play an important role in insulin resistance (98). Importantly, human and mouse resistin share only 59% identity (99), and humans lack one of the three murine resistin

isoforms. A very recent human study on circulating resistin levels showed no evidence of serum resistin mediating insulin resistance or in being a marker of obesity (100). This study showed no difference in resistin levels between normal-weight adolescents, obese insulin resistant nondiabetic, and type 2 diabetic adolescents even though there were significant differences in insulin levels. Women had approximately 20% higher levels of resistin than men, but these levels were not affected by fasting or leptin administration. Additionally, in humans, the resistin gene is on chromosome 19p13.3, a region not associated with obesity or insulin resistance (101). However, the downstream role of resistin in humans may still be important in the progression of type 2 diabetes, which is why receptor identification and the signaling pathway need to be defined.

V. Animal Models of Obesity

There are several animal models of obesity. Some commonly used models include the *db/db* and *ob/ob* mice and the fatty Zucker rat. A common feature of these models is that they either lack leptin protein (*ob/ob* mouse) or a functional leptin receptor (*db/db* mouse and fatty Zucker rat), thereby rendering them resistant to leptin. In the study of wound healing, the *db/db* and *ob/ob* mice have been used extensively while the fatty Zucker has not. The use of rats in wound healing rather than mice has several advantages; a larger size allows for more wounds on a single animal and therefore more controls for each test wound. The size also allows for larger wound areas so that several wound phases can be readily observed. Finally, the skin of the rats is substantially thicker than mouse skin and therefore closer histologically to human skin.

Despite the advantages of using rats, mice are often used to study wound healing due to the variety of strains available, the cost efficiency of housing and breeding and, because they have been so well studied, background data are abundantly available.

A. *db/db* and *ob/ob* Mice

The *ob/ob* mice lack leptin protein (102). These mice exhibit marked obesity, hyperphagia, transient hyperglycemia and very high plasma insulin concentrations, associated with an increase in the size and number of beta cells in the islets of Langerhans (103, 104). The C57B6/J *ob/ob* mouse expresses a 20-fold increase in leptin (*ob*) mRNA. However, there is a nonsense mutation resulting in an arginine changed to a stop codon at position 105 which prevents the production of leptin protein. The SM/Ckc-$+^{Dac}ob^{2J}/ob^{2J}$ mouse does not synthesize leptin mRNA due to a retroviral-like transposon in the first intron of the *ob* gene (105). This genomic insertion disrupts the expression of leptin mRNA.

The *db/db* mouse is characterized by obesity, hyperphagia, and severe diabetes with hyperglycemia (106, 107). In these mice, plasma insulin begins to rise at 10 days of age, peaks at 6-10 times normal by 2-3 months, then falls to near normal levels. The beta cells are hyperplasic and hypertrophic when insulin levels are high, and become atrophied when insulin levels drop. At this time glucose levels rise until death at 5-8 months. The autosomal recessive mutation (*db*) was first detected in C57BL/KsJ mice and was mapped to the middle of chromosome 4 (107). Since then it has been detected in at least four other mouse strains and two rat strains (108, 109). It is important to note that

although the mutation is similar in multiple mouse and rat strains, because of the difference in genetic background, the severity of disease may manifiest differently in each.

B. Fatty Zucker Rat

The fatty Zucker (fa/fa) rat exhibits leptin resistance resulting from a nucleotide substitution at position 880 (A→C) causing an amino acid substitution at position 269 (Gln→Pro) (110). Genetic mapping of chromosome 4 shows that fa and *diabetes* (*db*) are homologous loci on the rat and mouse genomes respectively. However due to the nature of the leptin receptor mutation in the fatty Zucker, there is still some residual leptin action. These rats are moderately insulin resistant but retain some insulin-mediated peripheral glucose uptake (111). These rats are obese, hyperinsulinemic, and glucose intolerant, yet normoglycemic (112).

C. JCR:LA-cp Rat

The development of the JCR:LA-cp rat strain is described in Russell et al. (113). The JCR:LA-cp rat incorporates the corpulent gene that was identified by Koletsky (114). The phenotype of these rats is due to a Tyr763 stop mutation in the extracellular domain of the leptin receptor. This results in a truncated protein unable to insert into the cell membrane as it lacks both a transmembrane and intracellular domain. The obese rats are homozygous for the cp gene and lean rats are either heterozygous (cp/+) or wild-type (+/+). These rats are obese by 3 weeks of age, hyperinsulinemic by 4 weeks and insulin

resistant at 8 weeks. By 12 weeks of age, the glucose uptake and turnover in the male cp/cp rats is no longer responsive to insulin. Male cp/cp rats begin to develop atherosclerotic lesions at 6 months and by 9 months, 100% of the male animals exhibit advanced lesions in the aortic arch.

The male cp/cp rats develop more severe atherosclerosis and myocardial lesions than females, these lesions are strongly correlated with hyperinsulinemia (115). The male rats also exhibit a more severe hyperplasia of the B cells in the pancreas and this results in hyperinsulinemia and impaired glucose tolerance (116). Cp/cp rats have elevated levels of triglyceride rich very low density lipoprotein (VLDL) resulting in hypertriglyceridemia, this is more extreme in the female cp/cp (117). Low density lipoprotein (LDL) and high density lipoprotein (HDL) are also elevated resulting in high concentrations of cholesterol and cholesteryl esters. Female cp/cp rats may not experience atherosclerosis to the same degree as male rats due to differences in sex-linked hormone levels.

Unlike the JCR:LA-cp/cp rat, other rat models used to study obesity and insulin resistance such as the fatty Zucker do not develop cardiovascular disease. Metabolic syndrome in humans is associated with abdominal obesity, mild type 2 diabetes and high risk for cardiovascular disease. Obese and insulin resistant individuals are also prone to chronic wounds.

VI. Adipokines and Wound Healing

The wound healing process is extremely intricate. Add to this balance the involvement of adipokines due to obesity and insulin resistance and the problem becomes increasingly complex. How much influence do the products of adipocytes have on healing? It is likely that there is some involvement since the adipose tissue is in close proximity to the overlying dermis. Leptin receptors, including the long form, have been identified on dermal fibroblasts (118). It has also been demonstrated that fibroblasts secrete leptin protein and levels are increased in the presence of insulin. Fibroblast-derived leptin may affect collagen and cytokine synthesis and aid in wound repair. TNF-α is a cytokine that may impede wound healing by negatively regulating TGF-β1 receptor signaling (119) and inhibiting the induction of type I collagen by TGF-β1 (120). TNF-α induces Smad7 and suppresses TGF-β1/Smad signaling by occupying sites on TGF-β1 type I receptor thereby inhibiting the phosphorylation and nuclear translocation of Smads involved in TGF-β1 signaling. The characterization of adipokines is a relatively new field of study and their impact on wound healing is yet to be defined.

VII. FIGURES AND LEGENDS

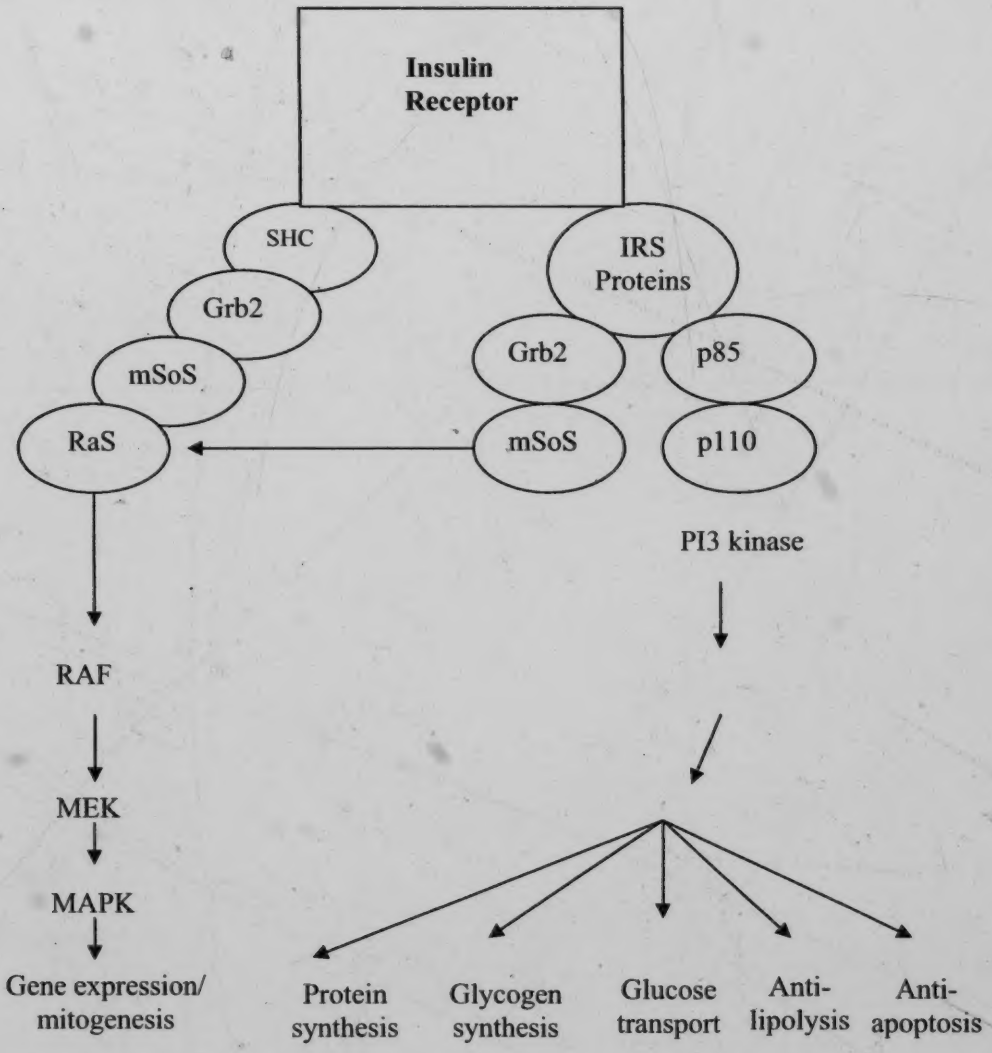
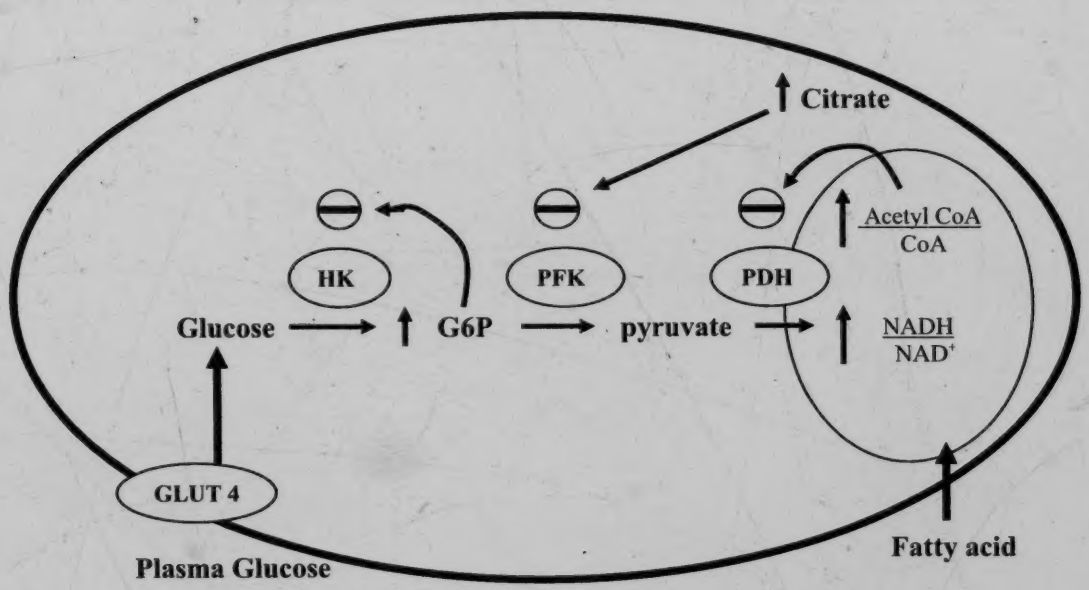


Figure 3-1

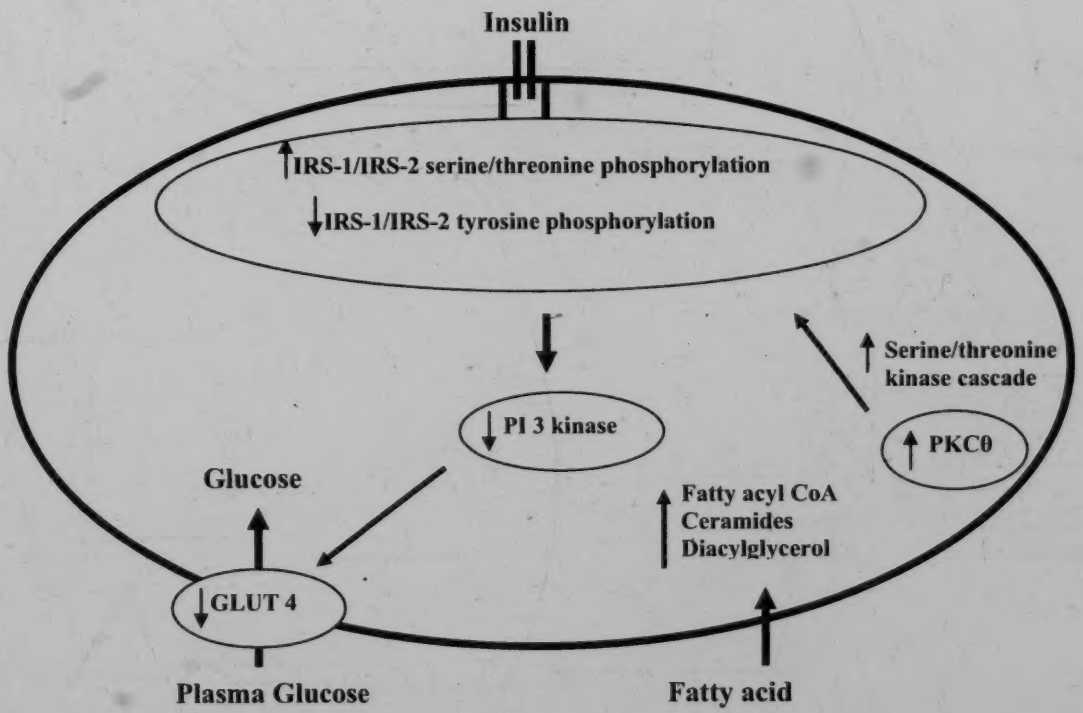
Insulin signal transduction (reviewed in12, 121) is initiated following insulin binding to its receptor (IR) which is a transmembrane tyrosine kinase in a $\alpha 2\beta 2$ tetramer configuration. Phosphorylation of IR results in the activation of insulin receptor substrate-1 (IRS-1) proteins, which contain a tyrosine phosphate binding domain. The phosphotyrosines on IRS-1 serve as high affinity binding sites for the docking and activation of intracellular signaling proteins. Two major signaling pathways are activated through the insulin receptor. The mitogen activated protein (MAP) kinase pathway is primarily responsible for the growth-promoting effects of insulin and the PI3-K pathway mediates the metabolic responses to insulin. Each of these pathways may interact and activate the other under specific circumstances. Modified from: Le Roith and Zick (121).

Figure 3-2

A


Citrate
HK
PFK
PDH
Acetyl CoA
CoA
Glucose
G6P
pyruvate
NADH
NAD+
GLUT 4
Plasma Glucose
Fatty acid

B

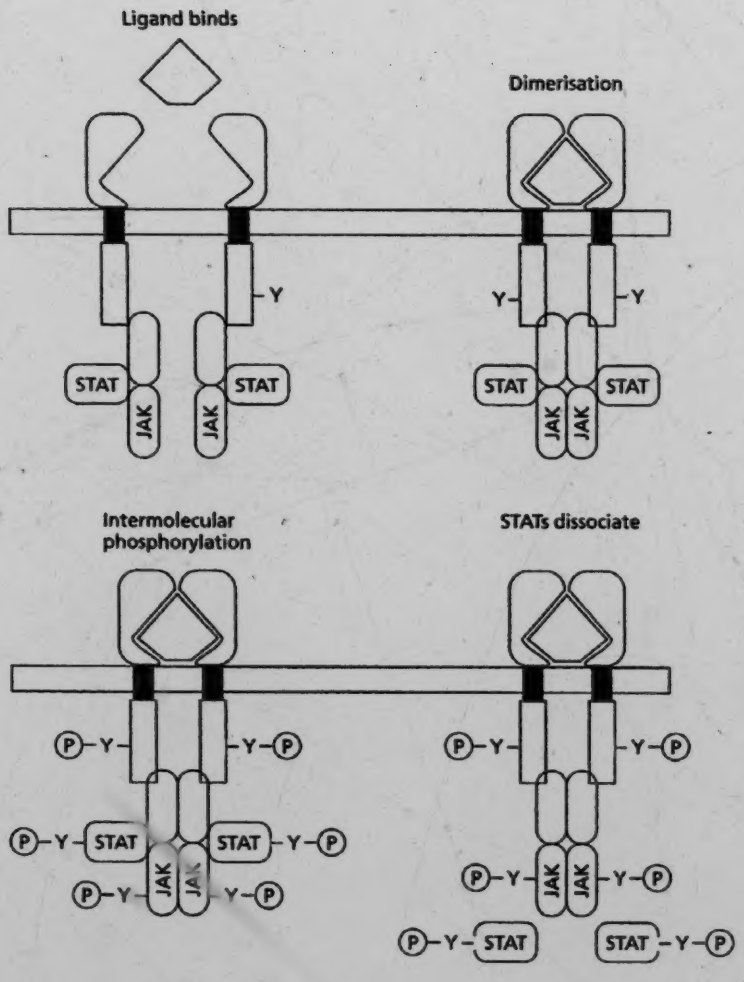

Insulin
IRS-1/IRS-2 serine/threonine phosphorylation
IRS-1/IRS-2 tyrosine phosphorylation
Serine/threonine
kinase cascade
PI 3 kinase
PKCθ
Glucose
Fatty acyl CoA
Ceramides
Diacylglycerol
GLUT 4
Plasma Glucose
Fatty acid

(Reproduced from 122)

A) The mechanism of fatty acid-induced insulin resistance in skeletal muscle as proposed by Randle *et al.* (17). An increase in fatty acid concentration results in an elevation of the intramitochondrial acetyl CoA/CoA and NADH/NAD^+ ratios, with subsequent inactivation of pyruvate dehydrogenase. This in turn causes citrate concentration to increase, leading to inhibition of phosphofructokinase. Subsequent increases in intracellular glucose-6-phosphate concentration would inhibit hexokinase II activity, which would result in an increase in intracellular glucose concentration and a decrease in muscle glucose uptake.

B) Proposed alternative mechanism for fatty acid-induced insulin resistance in human skeletal muscle. An increase in delivery of fatty acids to muscle or a decrease in intracellular metabolism of fatty acids leads to an increase in intracellular fatty acid metabolites such as diacylglycerol, fatty acyl CoA, and ceramides. These metabolites activate a serine/threonine kinase cascade (possibly initiated by protein kinase Cθ) leading to phosphorylation of serine/threonine sites on insulin receptor substrates (IRS-1 and IRS-2), which in turn reduces the ability of the insulin receptor substrates to activate PI 3-kinase. As a consequence, glucose transport activity and other events downstream of insulin receptor signaling are diminished. HK, hexokinase II; PFK, phosphofructokinase; PDH, pyruvate dehydrogenase; PKCθ, protein kinase Cθ.

Figure 3-3



(Reproduced from 123)

Summary of cytokine receptor signaling via the JAK/STAT pathway. Dimerisation of the receptors by ligands occurs. This induces intermolecular phosphorylation of JAK kinases associated with the cytoplasmic domains. Activated JAK kinases phosphorylate STAT substrates which dissociate from the receptor complex.

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CHAPTER 4

The JCR:LA-cp Rat: A Novel Model for Impaired Wound Healing

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I. ABSTRACT

The JCR:LA-cp obese rats and their lean controls were evaluated as a type 2 diabetic wound healing model and the healing quality was characterized. This model of insulin resistance has been used extensively to study atherosclerosis but has not previously been used to study wound healing. Six circular excisional wounds were made on the dorsum of each rat and followed to day 21. Tracings of the wounds were made and used to assess the rate of wound closure. Planimetry demonstrated a significantly diminished contraction of wounds in obese rats but no significant difference in reepithelialization was observed. Collagen content was estimated from the hydroxyproline content in wounded and unwounded skin. There were significantly lower levels of hydroxyproline in the wounds of obese, compared to lean animals at day 21. Histology demonstrated adipose tissue in place of dermal tissue in the JCR:LA-cp/cp rat in both unwounded tissue and in the wound at day 21. Active TGF-β1 was measured in the serum using the plasminogen activator inhibitor-1/luciferase (PAI-1/L) and serum total TGF-β was measured using an ELISA. No significant differences were found between groups. Both active and total TGF-β were measured in tissue sections using the PAI-1/L assay. There was no significant difference in either active or total TGF-β between genotypes.

These results indicate a deficiency in wound healing in obese animals characterized by decreased wound contraction, decreased collagen production, and changes in histology. The JCR:LA-cp rat develops insulin resistance, atherosclerosis and

early Type 2 diabetes and may be a good model of the impairment of wound healing in humans with metabolic syndrome.

II. RATIONALE FOR CURRENT STUDY

Our current study will focus on wound healing of the obese JCR:LA-cp rat. Although this rat model has been used extensively to study atherosclerosis, its wound healing characteristics have not been assessed. We believe this model to be of interest due to its underlying condition. In addition to being obese, this rat model is also insulin resistant and unlike the fatty Zucker rat, experiences atherosclerosis. We plan to assess wound contraction, histology, hydroxyproline content and TGF-β1 concentrations in both serum and tissue sections. In this next study we may see differences in the obese JCR rat which will lead to more detailed studies including cell behaviour and treatment regimens to improve healing in these animals.

III. INTRODUCTION

The World Health Organization has recently declared obesity a worldwide epidemic. In the United States, approximately half the adult population is considered overweight, and of these, half are clinically obese. Clinically obese individuals have an increased risk of developing type 2 diabetes and have a mortality rate three times that of those with normal weight. Chronic skin ulcers have also been correlated with obesity (1). Leptin is an adipose-derived hormone, that circulates in plasma as a 16 kDa protein and plasma levels of leptin are highly correlated with adipose tissue mass (2). Administration of leptin causes a dose-dependent decrease in body weight in mice deficient in leptin

hormone (*ob/ob* mice), resulting from a reduction in adipose tissue, but not lean body mass (3). *Ob/ob* mice resemble starved animals in that they have decreased body temperature, hyperphagia, decreased immune function and infertility (4), all of which are reversed by administration of leptin. There are five splice variants of the leptin receptor gene (OB-R) which shows sequence homology to the cytokine receptor superfamily. Only the splice variant ObRb (also referred to as the long form) has been shown to mediate intracellular signaling through the activation of the Jak-STAT pathway (5).

The development of the JCR:LA-cp rat strain has been described (6). The JCR:LA-cp rat incorporates the corpulent gene that was isolated by Koletsky *et al.* (7). The *cp* gene has been shown to have a Tyr763 stop mutation in the extracellular domain of the long form of the leptin receptor (ObRb isoform), leading to the phenotype observed in the obese JCR rat (8). The obese rats are homozygous for the cp gene and lean rats are either heterozygous (cp/+) or wild-type (+/+). Homozygous animals become detectably obese by 3 weeks of age, mildly hyperinsulinemic by 4 weeks and highly insulin resistant at 8 weeks. By 12 weeks of age, glucose uptake and turnover in the male cp/cp rats is no longer responsive to insulin. Male cp/cp rats begin to develop atherosclerotic lesions at 6 months and by 9 months, 100% of the male animals exhibit advanced lesions in the aortic arch. Only the male cp/cp rats develop atherosclerosis and myocardial lesions which are strongly correlated with hyperinsulinemia (9). Unlike the JCR:LA-cp rat, other rat models used to study obesity and insulin resistance, such as the fatty Zucker rat, do not develop cardiovascular disease. Metabolic syndrome in humans is associated with abdominal obesity, mild type 2 diabetes and high risk for cardiovascular disease. Obese and insulin resistant individuals are also prone to chronic wounds. It is for these reasons

that we chose the JCR:LA-cp rat to study chronic wounds and possible modalities of treatment.

In this study, the obese JCR:LA-cp rat was examined as a type 2 diabetic impaired wound healing model. These animals exhibit impaired healing, characterized by decreased wound contraction and hydroxyproline content.

IV. MATERIALS AND METHODS

A. Animals

Male rats of the JCR:LA-cp strain, obese (cp/cp) and lean (cp/+ or +/+), were raised in an established breeding colony at the University of Alberta as previously described (10). The rats were weaned at 3 weeks of age and housed initially in pairs in polycarbonate cages on woodchip bedding at 20°C and 55% relative humidity. The lighting was on a 12-hour cycle. At 6 weeks of age the rats were housed individually. Food was available at all times (Teklad Rodent Diet; Harlan Sprague-Dawley, Madison, WI). This is a corn and wheat-based diet of less than 4% total lipid and 23% protein content; the energy content is approximately 3.3 kcal/g.

B. Wound Generation

The rats were premedicated with buprenorphine 0.03mg/kg (Buprenex™, Reckitt & Coleman, Richmond VA. USA), and glycopyrulate™ 0.1mg/kg (Sabex Inc. Boucherville, Que., Canada). Surgical plane anesthesia was induced and maintained by facemask using isoflurane™ (Biomeda-MTC, Cambridge, ON) and O_2 to effect. The

dorsal surface was prepared with 70% ethanol and 10% Betadine™, (Purdue Fredrick Inc. Toronto, Ont., Canada). Six full thickness excisional wounds were made on each rat (3 wounds on each side of the midline) using a 6 mm Accu Punch™ disposable biopsy tool (Dormer Laboratories, Mississauga, Ont. Canada). There was no experimental intervention in the wounds and six animals from each experimental group were sacrificed at day 21. Wound tracings were performed on transparency film to assess wound reepithelialization and contraction. Transparencies were scanned and planimetry was performed using Scion Image (NIH, USA) with a scale on each transparency for standardization. For the initial surgery (day 0) and at termination, samples were collected for histology (H&E), immunohistochemistry (TGF-β1), hydroxyproline content, and the plasminogen activator inhibitor/luciferase assay. Samples for histology were preserved in 4% paraformaldehyde, and samples for hydroxyproline were snap frozen and stored at –80°C. At termination, serum was collected for analysis of TGF-β1 content by ELISA.

C. Hydroxyproline Analysis

The content of collagen in unwounded and wounded tissue was determined by mass spectrometric analysis for 4-hydroxyproline (11). Skin samples (6 mm biopsies) from lean and obese rats were freeze-dried. Internal standard (*N*-methyl-L-proline) and 6 N HCl solution was added to wound tissue, and each sample was then hydrolyzed overnight at 115 °C. The *O*-butyl ester derivatives were prepared with 10% BF_2-butanol for 30 min at 120 °C after drying the hydrolysate. Liquid chromatography (using a Eclipse XDB-C18 column)/mass spectrometry analysis was performed on a Hewlett-

Packard (series 1100, Atlanta, GA) mass selective detector monitoring the ions at m/z 188 and 186.

D. TGF-β1 ELISA

To determine the concentration of total TGF-β1 in rat serum, a sandwich ELISA based on the procedure reported by Danielpour *et al.* (12) was used. Serum was stored frozen at -80°C until assayed. Briefly, 96-well plates were coated with 100 µL per well of mouse monoclonal antibody to human TGF-β (R&D Systems, Mineapolis, MN) at a concentration of 1 µg/mL in phosphate-buffered saline (PBS). The plates were incubated for 3 h at room temperature (RT) followed by 16 h at 4°C. After washing twice with PBS-Tween-20 (PBS-T), the plates were blocked with 1% bovine serum albumin (BSA, crystallized, Sigma) for 60 min at RT and washed twice with PBS-T. Rat serum was acidified with 12 µL/500 µL of 3N HCl for 15 min at RT and neutralized with 35 µL/500 µL of 1M HEPES/5N NaOH (5:2). One hundred microliters per well of the acidified/neutralized samples was added and the plates were then incubated at 37°C for 1 h. After washing, the plates were incubated with 100µL per well of chicken anti-human TGF-β IgG (R&D Systems) at a concentration of 2.5 µg/mL for 1 h. After washing five times with PBS-T, the plates were incubated with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) at RT for 1 h followed by washing 5 times with PBS-T. After addition of the substrate (o-nitrophenyl phosphate, 1.5 mg/mL, Sigma), the plates were incubated at RT for 1 h and the optical density was read using a THERMOmax (Molecular Devices, Minlo Park, CA) microplate reader at a wavelength of 405 nm.

Serial dilutions (0, 125, 250, 625, 1 250, to 2 500 pg/mL) of recombinant human TGF-β1 (R&D Systems) were used to prepare a standard curve.

E. Luciferase (PAI/L assay)

The plasminogen activator inhibitor-1/ luciferase assay was performed as described by Yang *et al* (13). Briefly, cryosections were cut 20 μm thick onto 12 mm circular coverslips (Fisher Scientific) and placed into 24 well plates with the section facing up. 500 μL of 0.1% pyrogen-poor BSA (w/v) (Pierce, Rockford, IL) in serum-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) was added to each well. TGF-β1 was activated by acidifying each well with 3N HCl for 15 min followed by neutralization with 5N NaOH/HEPES. TGF-β1 was eluted from sections overnight and media was then assayed for TGF-β1 bioactivity using mink lung epithelial cells (MLEC) (a generous gift from Dr. Daniel Rifkin, New York University Medical Center, New York, NY) bearing the plasminogen activator inhibitor-1 promoter-luciferase (PAI/L) construct (14). Active TGF- β1 in serum was also assayed using this method due to its high sensitivity. Serum was diluted 1:1 in 0.1% pyrogen-poor BSA (w/v) (Pierce, Rockford, IL) in serum-free DMEM and added to MLECs. MLECs transfected with this construct were cultured in DMEM supplemented with 10% FBS, L-glutamine (2mM), penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL), and amphotericin B (250 ng/mL). A standard curve was constructed (0-500 pg/mL) using recombinant human TGF-β1 (R&D Systems) diluted in 0.1% BSA in DMEM.

Transfected MLECs were plated at a density of 1.6×10^4 cells/well in 96-well tissue culture plates. The cells were allowed to attach for 3 h at 37°C in an atmosphere of 5%CO_2. Cells were washed with PBS and the medium was replaced with conditioned media, serum samples or standards and incubated for 14 hours at 37 °C. Cells were then washed with PBS and lysed using 50 μL of 1× cell lysis buffer (Analytical Luminescence, San Diego, CA) for 20 min at room temperature on a rotating platform. The cell lysates (45 μL) were then transferred to clear polypropylene scintillation vials into which 100 μL of both substrate A and B (Analytical Luminescence) were added and samples were immediately analyzed using the Beckman LS 6000 TA scintillation counter. The tissue TGF-β1 measurements were corrected for tissue volume by measuring the tissue area using a grid micrometer and multipying the area by the tissue thickness (20μm). The concentration of TGF-β1 per section was expressed as pg/mm^3 tissue.

F. Statistical Analysis

A two-tail Students unpaired t-test was used for all statistical analyses using GraphPad InStat 3 (San Digeo, USA). $P<0.05$ was considered statistically significant.

V. RESULTS

In loose-skinned animals, healing occurs primarily by wound contraction, and then by reepithelialization, via migration and proliferation of keratinocytes. Contraction and reepithelialization were measured by tracing the original boundary and the open area

of the wounds respectively. Wounds from obese animals (Fig 4-1 d and f) showed a much larger original wound boundary compared to those from lean control animals (Figure 4-1 c and e), indicating less contraction. The rate of reepithelialization was not significantly different between control and obese animals at day 9 when scabs were removed (Fig 4-1 c and d). Wound contraction was diminished in obese animals compared to lean controls as measured by planimetry (Fig 4-2), at day 1(1.27 ± 0.16 SD vs 0.92 ± 0.10 SD, $P<0.005$) and day 21(0.74 ± 0.12 SD vs 0.300 ± 0.069 SD, $P<0.0001$).

Hydroxyproline was used as a measure of collagen content as it comprises approximately 10% of collagen. The level of hydroxyproline in the unwounded skin was not significantly different between genotypes (244.51 ± 19.77 SD vs 270.58 ± 24.25 SD μg/biopsy, for obese and lean rats respectively), however, there was a significant difference in the wounds of obese compared to lean animals at day 21 (145.77 ± 16.12 SD vs 200.04 ± 9.84 SD μg/sample, $P<0.0001$) (Fig 4-3).

Histologic examination demonstrated adipose tissue infiltration of the dermis in the obese JCR:LA-cp rat in both unwounded tissue and in the wound at day 21, resulting in a much thinner dermis in the obese JCR rat (Fig 4-4). The extracellular matrix was noticeably looser in the obese rat skin compared to the normal lean control.

TGF-β1 is a fibrogenic growth factor that plays an important role in wound healing. We found no significant differences in serum active (lean=82.84 ± 10.17, obese=99.84 ± 20.03) or latent (lean=284.62 ± 98.61, obese=284.55 ± 54.38) TGF-β1.

Nor did we measure significant differences in active (lean=34.35 ± 4.25, obese=39.02 ± 30.28) or latent (lean=89.92 ± 22.06, obese=115.65 ± 31.75) TGF-β1 in day 21 wound tissue.

VI. DISCUSSION

Impaired wound healing in patients with clinical obesity and type 2 diabetes is a serious multifactorial problem that has been studied with various animal models. Defective wound healing has been described in *db/db* mice. As in the JCR:LA-cp rat, these mice also exhibit defective leptin signaling, due to a mutation in ObRb. The impairment of wound healing in the *db/db* mouse is characterized by a delay in cellular infiltration, granulation tissue formation and reepithelialization, as well as reduced angiogenesis and collagen production (15,16). The contraction of wounds in *db/db* mice has been shown to be less than in non-diabetic littermates (17). An estimated 90% of wound closure was due to contraction in non-diabetic mice whereas only 40% was due to contraction in diabetic mice. Wounds in non-diabetic mice appeared to contract rapidly with granulation tissue and re-epithelialization occurring in a small region. In contrast, the wounds in *db/db* mice filled in primarily by granulation tissue formation and re-epithelialization.

Leptin has been shown to act as a mitogen for epithelial, endothelial, and pancreatic cells *in vitro* (18,19,20). The influence of leptin on keratinocytes during reepithelialization was studied in *ob/ob* mice, which do not produce leptin protein (18). Leptin improved reepithelialization of excisional wounds and accelerated wound healing

in *ob/ob* mice. Animals with leptin-treated wounds exhibited a highly organized epithelium whereas the phosphate-buffered saline-treated wounds were disorganized and atrophied.

In our model, we did not observe a significant delay in closure of wounds in obese rats. This may be due to the fact that scabs were removed at day 9 when most wounds were closed. Earlier removal of scabs would have disrupted the wound edges. The most dramatic difference between wounds in obese and lean animals was the diminished contraction noted in the former. It is possible that decreased fibroblast contraction occurs as a downstream effect of defective leptin signaling. Future experiments will focus on the potential role of leptin on fibroblast behavior.

In our study, we did not measure significant increases in serum or wound TGF-β1 in obese animals. It may be that the absence of other growth factors plays a greater role in the healing of obese JCR rats. Alternatively, had we tried to measure tissue TGF-β at earlier time points during healing, we may have noted differences. Previous studies have reported impaired reepithelialization with increased TGF-β levels in the skin (21,22,23). However, in our model, early removal of the scab would have caused mechanical damage and therefore at day 9 we may have missed any differences in reepithelialization. Increased levels of TGF-β1 are found in sepsis and following trauma, and this increase correlates with immunosuppression, due in part to a reduction in lymphocyte proliferation

(24,25). This phenomenon also occurs in patients with anorexia nervosa (26), which, notably, is also associated with decreased leptin levels.

The mechanisms of the immune response are now clearer since the identification of CD4+ T helper subpopulations (Th1 and Th2). Th1 cells secrete proinflammatory cytokines (i.e. IL-2 and IFN-γ, and lymphotoxin) and are involved in macrophage and monocyte-mediated responses. Th2 cells secrete regulatory cytokines (i.e. IL-4, IL-5, IL-6, IL-9, and IL-10) and regulate antibody production, mast cell and eosinophil proliferation and function. Leptin is capable of increasing Th1 while suppressing Th2 cytokine production (27). It has been suggested that suppressed cell-mediated immunity during nutritional deprivation is due to a decrease in leptin expression. In obesity, where leptin resistance occurs, individuals have an increased susceptibility to infection. In wound healing, it has been demonstrated that a severe injury also results in a decreased Th1 response (28). This T cell imbalance leads to generalized immunosuppression and increased susceptibility to infection and sepsis. It is therefore likely that a deficiency of leptin combined with a severe injury would cause substantial immunosuppression and result in wound healing deficiencies. Interestingly, the decreased Th1 response observed with defective leptin signaling corresponds to a decrease in interferon-γ (IFN-γ). IFN-γ -/- mice have been shown to produce more TGF-β (29). Also, IFN-γ has been shown to regulate TGF-β signal transduction (30). Therefore TGF-β signaling may not be effectively regulated in this model leading to autoinduction of TGF-β.

Our results demonstrate an impairment of wound healing in the obese JCR rat that has not previously been described. We found similarities between our model and the *db/db* mouse model with regards to decreased collagen content and a significant decrease in wound contraction. In contrast to the *db/db* mouse, we did not see a significant decrease in the rate of reepithelialization in the obese JCR rat. This may be due to differences in the severity of disease between models, the *db/db* mouse becomes hyperinsulinemic and hyperglycemic while the obese JCR:LA-cp rat becomes hyperinsulinemic but maintains normal glucose levels. These differences may indicate that the fibroblasts play a major role in the wound healing impairment in this model.

VII. FIGURE AND LEGENDS

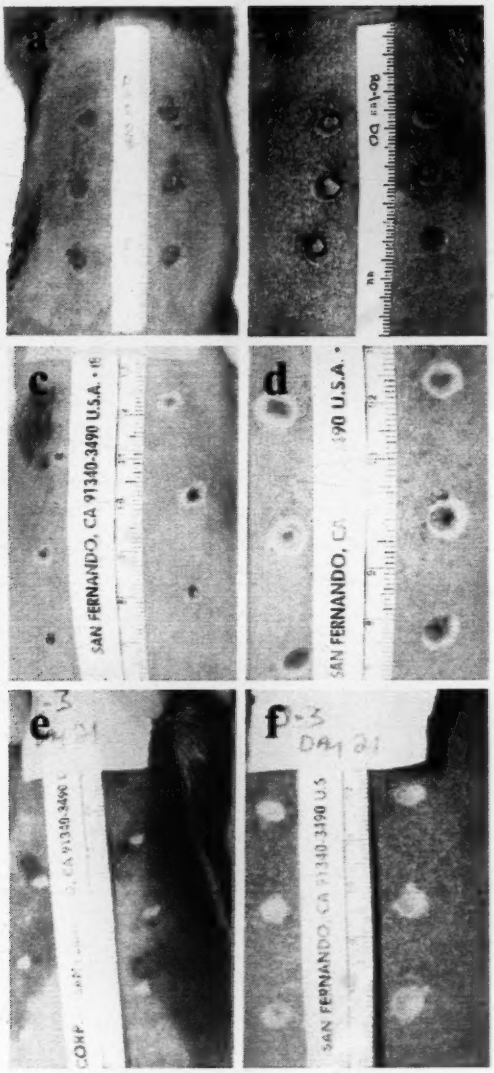

Figure 4-1

Wound appearance on obese and control rats on various days postwounding: (a,b) Wounds at Day 0, (c,d) day 9, and (e,f) day 21 wounds. Lean rats (a,c,e) and obese (b,d,f) JCR rats.

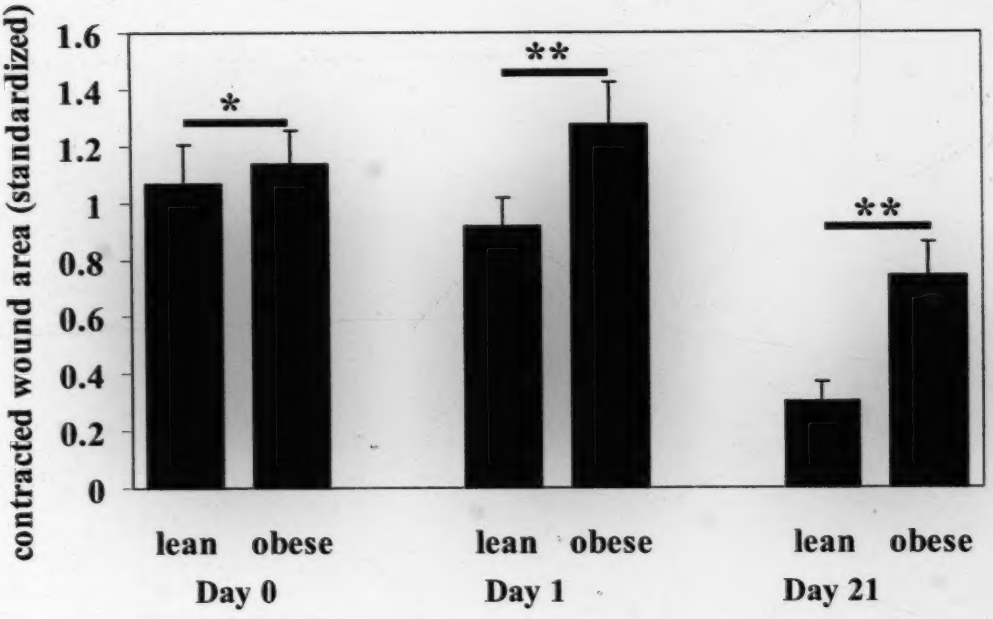

Figure 4-2

Comparison of contracted wound areas: The original boundary of each wound was traced and scanned to measure wound contraction. Image analysis was performed to measure standardized wound area. O=obese, L=lean. *P<0.005, **P<0.0001 (n=6).

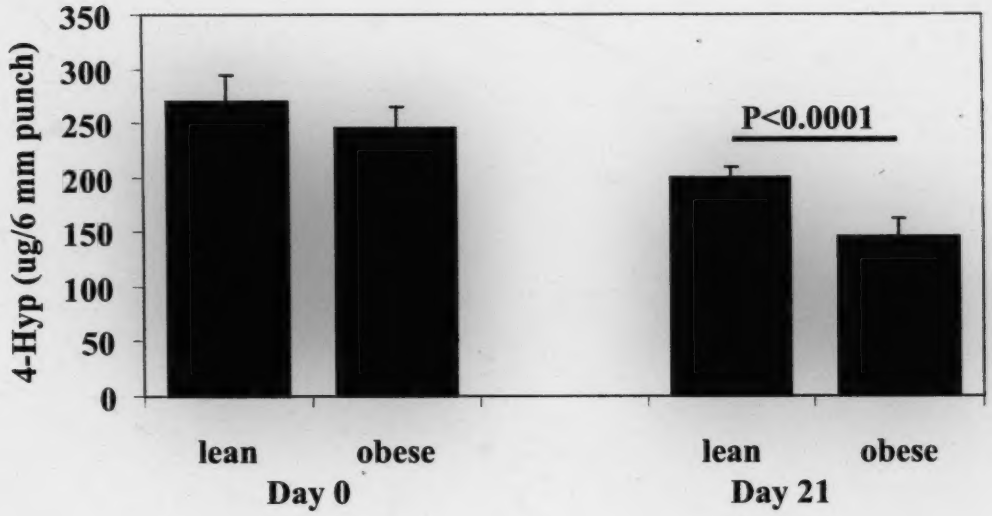

Figure 4-3

Hydroxyproline analysis of wounds: Hydroxyproline content from 6 mm punch biopsies. n=6.

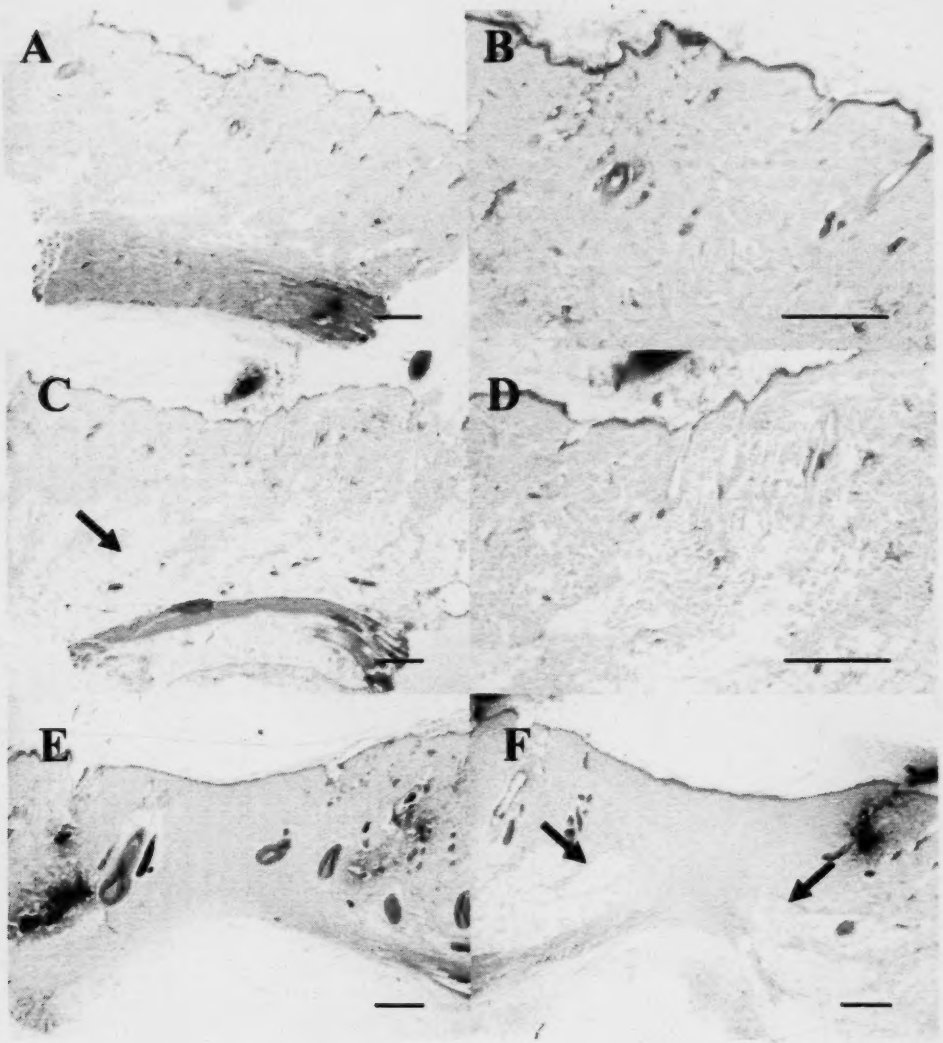
A
B
C
D
E
F

Figure 4-4

Rat skin histology: Histology of unwounded skin for lean (A) and obese rats (C) using low power magnification demonstrates the replacement of dermis with adipose tissue in obese rats. High power magnification demonstrates the loose extracellular matrix in obese rat skin (D) compared to lean (B). Low power magnification at day 21 demonstrates the presence of adipose tissue in the wound area of obese rats (F) (arrow) which is absent in lean rats (E). Low magnification (A, C, E, F) is 10x, high magnification (B, D) is 20x, scale bar = 500 μm.

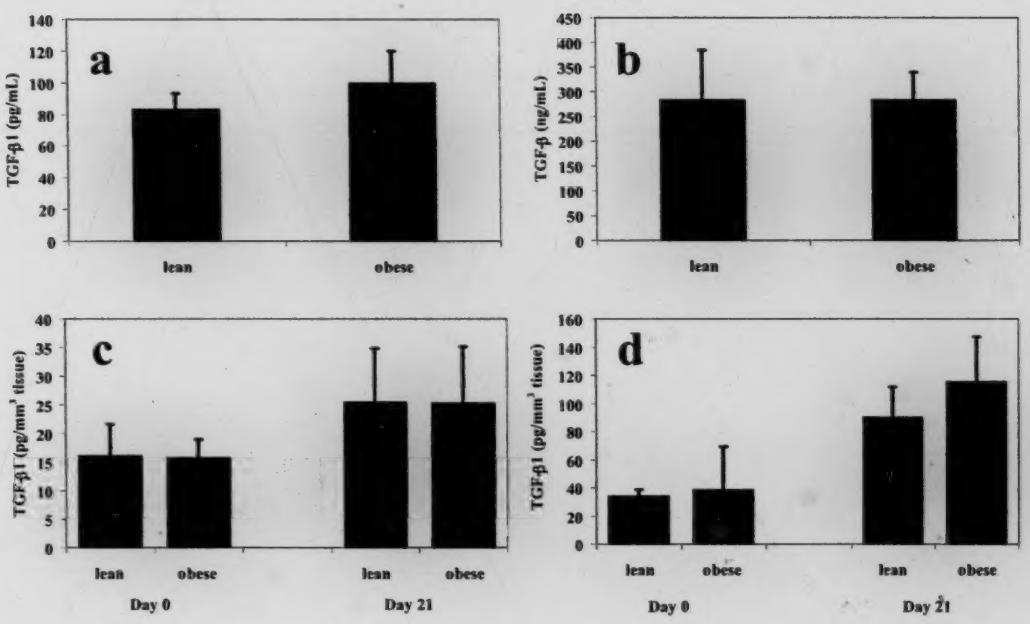

Figure 4-5

Transforming growth factor-β1 in serum and skin: (a,b) Transforming growth factor-β1 in serum and in (c,d) unwounded and wounded skin. Serum active TGF-β1 measured using the PAI-1/L assay (a) and total serum TGF-β1 was measured using an ELISA (b). (n=6-9 in triplicate). Active (c) and total (d) TGF-β1 in unwounded and wounded rat skin was measured using the PAI-1/L assay. (n=6, in quadruplicate).

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Chapter 5

Characterization of JCR:LA-cp Fibroblasts

I. ABSTRACT

Fibroblasts play an integral role in wound healing. Dermal fibroblasts secrete numerous growth factors, are involved in wound contraction and synthesize extracellular matrix proteins such as collagen. In a previous study, we demonstrated a decrease in wound contraction in the obese JCR:LA-cp rat, a novel animal model for wound healing, compared to lean controls, as well as a decrease in hydroxyproline content (a constituent of type I collagen) of the obese rat wounds. We explanted fibroblasts from these animals to determine whether the absence of leptin signaling in the obese JCR:LA-cp rat modulates fibroblast behavior.

We found no significant differences in the size and proliferation of fibroblasts from obese rats compared to lean controls. We also noted no significant difference in TGF-β1 concentration of conditioned media, contraction of fibroblast populated lattices or expression of type I procollagen mRNA. We did however measure a significant decrease in hydroxyproline content in the conditioned medium collected from obese rat fibroblasts compared to lean ($P<0.0118$, obese 72.59 ± 10.70 vs. lean 100.00 ± 19.05).

This study demonstrates that although clear differences in healing were observed between lean and obese JCR:LA rats *in vivo*, these differences were either not due exclusively to fibroblast behaviour, or that the fibroblasts were not activated appropriately in this model as they would be *in vivo*.

II. RATIONALE FOR CURRENT STUDY

In the previous study, we demonstrated a significant decrease in wound contraction and hydroxyproline content in the wounds of obese JCR rats compared to lean controls *in vivo*. We hypothesize that these differences are primarily due to differences in fibroblast behavior. The current study will focus on the effect of the leptin receptor defect on the behavior of explanted fibroblasts. We performed a series of experiments on fibroblasts explanted from lean and obese rats including; fibroblast proliferation, cell size, TGF-β1 and hydroxyproline measurement from conditioned medium, type I procollagen mRNA, and fibroblast populated collagen lattice contraction. The results of this study will help to further characterize the wound healing defect of this novel chronic healing model.

III. INTRODUCTION

In a previous study, we observed deficient healing in the JCR:LA-cp rat, a novel animal model for defective wound healing in obesity (1). These obese rats exhibited decreased wound contraction, decreased hydroxyproline content in the wounds at day 21. As the deficiencies noted in the healing of obese animals appear to be related to fibroblast behavior, we explanted fibroblasts from these animals to determine whether the absence of leptin receptors modulates fibroblast behavior.

Most studies to date have concentrated on the effect of leptin signaling on keratinocyte behaviour. Leptin has been shown to act as a mitogen for epithelial, endothelial, and pancreatic cells *in vitro* (2-4). Fibroblasts have been shown to express leptin receptors and produce leptin protein (5). The leptin synthesis and secretion by

fibroblasts was shown to be regulated by insulin. This evidence led us to study the involvement of fibroblasts in the healing of JCR:LA-cp rats, a model in which the Ob-Rb leptin receptor is mutated such that the protein is truncated. The transmembrane and intracellular domains are not expressed and, as a result, the extracellular portion of the receptor is not inserted in the membrane. Based on our previous results, we hypothesize that in the absence of functional leptin receptors, the defective healing of the obese JCR:LA-cp rat is primarily due to alterations in fibroblast behaviour.

IV. METHODS

A. Cell Cultures

Fibroblast cultures were established using unwounded skin from obese and lean JCR:LA-cp rats. Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), as previously described (6). Three different strains of fibroblasts from obese and lean rats were used, all were at passages 3-5. Each experiment was duplicated.

B. Proliferation Assay

Fibroblasts were plated at a density of $1x10^5$/well in 6 well plates in triplicate. Cells were grown in 2% dFBS and each well was counted in triplicate using a Coulter Counter after 1, 2, 4, 7, 9, 11, 14, and 16 days in culture.

C. Cell Size Comparison

Latex beads (Sigma) 21.5 μm in diameter were used to calibrate the Coulter Counter instrument. The diameter threshold was then increased in steps from 11 μm to 29 μm. Samples were counted in triplicate at each gated threshold.

D. TGF-β1 ELISA

To determine the concentration of TGF-β1 in conditioned medium, a sandwich ELISA was used which was based on the procedure reported by Danielpour et al. (7). Cells were plated at a density of $5x10^5$/well in 6 well plates in triplicate. After 24 hours, medium was changed from 10% FBS in DMEM to 2% dialyzed FBS (dFBS) in DMEM and cells were incubated 48 hours. Media were collected and frozen at -20◦C until assayed and cells were counted manually. Briefly, 96-well plates were coated with 100 μL per well of mouse monoclonal antibody to human TGF-β (R&D Systems, Mineapolis, MN) at a concentration of 1 μg/mL in phosphate-buffered saline (PBS). The plates were incubated for 3 h at room temperature (RT) followed by 16 h at 4°C. After washing twice with PBS-Tween-20 (PBS-T), the plates were blocked with 1% bovine serum albumin (BSA, crystallized, Sigma) for 60 min at RT and washed twice with PBS-T. Rat serum or culture media from *in vitro* experiments were acidified with 12 uL/500 μL of 3N HCl for 15 min at RT and neutralized with 35 μL/500 μL of 1M HEPES/ 5 N NaOH (5/2). One hundred microliters per well of the acidified/neutralized samples was added and the plates were then incubated at 37°C for 1 h. After washing, the plates were incubated with 100μL per well of chicken anti-human TGF-β (R&D Systems) at a concentration of 2.5 μg/mL for 1 h. After washing five times with PBS-T, the plates were incubated with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) at

RT for 1 h followed by washing 5 times with PBS-T. After addition of the substrate (o-nitrophenyl phosphate, 1.5 mg/mL, Sigma), the plates were incubated at RT for 1 h and the optical density was read using a THERMOmax (Molecular Devices, Menlo Park, CA) microplate reader at a wave length of 405 nm. Serial dilutions (0, 125, 250, 625, 1 250, to 2 500 pg/mL) of recombinant human TGF-β1 (R&D Systems) were used to prepare a standard curve.

E. RNA Extraction and Hybridization

Fibroblasts were plated at a density of $1x10^5$/well in 6 well plates in triplicate. Following a 48 hour incubation in 2% dialyzed FBS (dFBS), culture medium was removed and cells were lysed in 2 mL of guanidinium isothiocyanate (GITC) as previously described (8). Total RNA was extracted using the procedure of Chirgwin et al. (9), separated by electrophoresis, and blotted onto nitrocellulose filters. Filters were then baked under vacuum for 2 h at 80°C and prehybridized in a solution containing 50% formamide, 0.3 M sodium chloride, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1x Denhardt's solution (1x = 0.02% BSA, Ficoll, and polyvinylpyrrolidone), 0.005% salmon sperm DNA, and 0.005% poly (A) for 3-4 h at 45°C. Hybridization was performed in the same solution at 45°C for 16-20 h using cDNA probes for either human TGF-β1, type I collagen, collagenase, or 18S ribosomal RNA. The probes were labeled with ^{32}P-α-CTP (DuPont Canada, Streetsville, Mississauga, Ontario, Canada) by nick translation. Filters were initially washed at RT with 2x SSC (1x = 0.15 M sodium chloride, 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) for 30 min, then for 20 min at 65°C in 0.1x SSC and 0.1% SDS solution. Autoradiography was performed by exposing Kodak

X-Omat film to the nitrocellulose filters at -70°C in the presence of an enhancing screen. The cDNA probes for 18S ribosomal RNA and collagenase (American Type Culture Collection; Rockville, MD), were obtained from the American Type Culture Collection (Rockville, MD). Type I procollagen cDNA probe was a gift from Drs. G. Tromp, H. Kuivaniemi and L. Ala-Kokko, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA.

F. Hydroxyproline Analysis

Fibroblasts were plated in triplicate at a density of $5x10^5$/well in 6 well plates and allowed to attach overnight in DMEM with 10% dFBS. Medium was changed to 2 mL of DMEM containing 2% dFBS, 50 ug/mL ascorbic acid, 50 ug/mL B-aminopropionitrile, and 0.1 mmol/L proline. Cells were incubated 96 hours, after which conditioned medium was collected and hydroxyproline was determined by mass spectrometric analysis (10). Samples were freeze-dried. Internal standard (*N*-methyl-L-proline) and 6 N HCl solution was added, and each sample was then hydrolyzed overnight at 115 °C. The *O*-butyl ester derivatives were prepared with 10% BF_2-butanol for 30 min at 120 °C after drying the hydrolysate. Liquid chromatography (using a Eclipse XDB-C18 column)/mass spectrometry analysis was performed on a Hewlett-Packard (series 1100, Atlanta, GA) mass selective detector monitoring the ions at m/z 188 and 186.

G. Fibroblast-Populated Collagen Lattice Contraction

Fibroblast-populated collagen lattices were made using bovine type I collagen extracted as described by Volpin and Veis (11) using a modification of the procedure of

Bell *et al* (12). Fibroblasts ($5x10^5$/well) suspended in DMEM + 10% FBS were embedded within a collagen matrix consisting of 2.14 mg/mL bovine collagen, 350 µl 3x DMEM, 26 µl 0.4M NaOH, 125 ul FBS (for a final concentration of 10%) in 6 well tissue culture plates. Gels were incubated 24 hours after which the edges of the gels were released from the well walls to allow for contraction. Digital photos were taken at 0, 4, 8, 24, and 48 hours and the diameter of the contracted gels was measured.

H. Statistical Analysis

A two-tail Students unpaired t-test was used for all statistical analyses using GraphPad InStat 3 (San Digeo, USA). $P<0.05$ was considered statistically significant.

V. RESULTS

The proliferation rates of three strains each of lean and obese rat fibroblasts were assessed using a Coulter counter over a period of 16 days (fig 5-1). We found that although a trend was noted from day 7 to 16 in that the obese rat fibroblasts were not as proliferative as the lean, this trend did not reach statistical significance (lean 71008.15 ± 5396.11 vs. obese 56347.41 ± 10048.51 cells at day 16).

The size of the lean and obese rat fibroblasts was compared to determine if fibroblast size may be a factor in fibroblast function (fig 5-2). Standard latex beads (21.5 µm diameter) were used to calibrate the Coulter counter. We found no significant difference in the size of the fibroblasts after analyzing 3 different strains of lean and obese fibroblasts.

Conditioned medium was used to measure the production of active and latent TGF-β (fig 5-3). TGF-β measurements were normalized using cell counts. After two separate experiments consisting of three strains of lean and obese rats fibroblasts, we found no significant differences in the expression of active (fig 5-3a) (lean 82.35±18.81 vs. obese 84.45±36.55 pg/100, 000 cells) or latent TGF-β (fig 5-3b) (lean 789.81±137.04 vs. obese 881.24±226.58 pg/100, 000 cells).

Type I procollagen was analyzed using northern analysis with 18S as a loading control (fig 5-4a). After correcting for loading, we found no significant difference in type I procollagen mRNA expression after combining the results of two separate experiments each consisting of 3 strains of lean and obese fibroblasts (fig 5-4b) (lean 1.18±0.33 vs. obese 1.27±0.16 relative units).

Conditioned medium was used to measure the production of hydroxyproline (fig 5-5). Because the overall scale of the two separate experiments was disparate, we normalized by adjusting the average result of the lean fibroblasts by a factor to bring the average to 100. The obese fibroblast hydroxyproline average was also multiplied by the same factor in each experiment. The result indicated a statistically significant difference in the hydroxyproline production between the lean and obese rats (P=0.0118, lean 100.00±19.05 vs. obese 72.59±10.70 ng/100, 000 cells).

To determine if the behaviour of obese rat fibroblasts was the primary reason for the decreased wound contraction observed *in vivo*, we assessed the contractility of the fibroblasts with fibroblast-populated collagen lattices (fig 5-6). After releasing the gels from the well sides, we measured the diameter for a period of 48 hours and noted no significant differences in the diameter of the gels seeded with lean JCR rat fibroblasts vs. obese JCR fibroblasts (lean 1.16±0.27 vs. obese 1.42±0.43 cm at 48 hours).

VI. DISCUSSION

Fibroblasts are essential for wound healing due to the fibrogenic growth factors they synthesize, their role in wound contraction, and their ability to produce extracellular matrix proteins (ECM) such as collagen, fibronectin and proteoglycans. Our most important finding in this study was the lower hydroxyproline content of the conditioned medium from obese JCR rat fibroblasts compared to lean rat fibroblasts (P=0.0118, lean 100.00±19.05 vs. obese 72.59±10.70 ng/100 000 cells). However, we did not measure a significant difference in type I procollagen mRNA. This may be due to the mRNA from obese rat fibroblasts not being translated into protein or because of the ability of the LC/MS to measure hydroxyproline in the ng range and thus it is able to discern very small differences between treatments. Collagen synthesis requires fibroblasts and is highly dependent upon and fibrogenic growth factors such as TGF-β1 (13, 14) and IGF-1 (15). We did not measure a difference in TGF-β1 concentration in the conditioned medium of lean and obese JCR fibroblasts, therefore the decrease in hydroxyproline may be due to the deficiency of some other fibrogenic growth factor such as IGF-I or IGF-II,

an increase in a growth factor which negatively regulates TGF-β signaling (IFN-γ or TNF-α), or increased metalloproteinases none of which were assessed in this study.

The expression of insulin-like growth factor (IGF)-I and IGF-II during wound healing in *db/db* mice is delayed (16). While levels of IGF-I in the *db/db* mice only reached 50% of the control, IGF-II were four times greater by day 10. Usually, IGF-II is highly expressed in fetal tissue and declines after birth, however in diabetic animals IGF-II appears to play an important role. Other studies have shown that IGF-I combined with PDGF did not have an additive effect on wound healing, while IGF-II and PDGF did (17). This indicates different functions of IGF-I and IGF-II during wound healing, and it has been speculated that IGF-II may compensate for IGF-I, which may be sequestered by binding proteins. Also, apoptosis in the *db/db* mouse is delayed and the delay is reversed with IGF-II and PDGF combined therapy (18).

TNF-α (19, 20) and IFN-γ (21) are cytokines that may impede wound healing by negatively regulating TGF-β1 receptor signaling and inhibiting the induction of type I collagen by TGF-β1. TNF-α and IFN-γ both induce Smad7 and suppress TGF-β1/Smad signaling by occupying sites on TGF-β1 type I receptor thereby inhibiting the phosphorylation and nuclear translocation of Smads involved in TGF-β1 signaling.

Interestingly, although we observed less proliferation of fibroblasts from obese rats compared to lean, Absher et al. (22) observed the opposite in smooth muscle cells

(SMCs) isolated from obese JCR:LA-cp rat aortas, a cell type sharing a common stem cell origin to fibroblasts. Absher found that SMCs from obese JCR:LA-cp rats exhibited increased proliferation compared to lean control SMCs and proliferation of these cells was further increased with exposure to TGF-β, bFGF and IGF-1. The proliferative response of SMCs from obese JCR:LA-cp aortas may play an integral role in the development of atherosclerosis in these animals. The difference in proliferation between our study and Abshers' may be due to the inherent differences in cell types. We would expect an increase in proliferation of the obese rat fibroblasts in the presence of exogenous fibrogenic growth factors, but whether the proliferation of obese rat fibroblasts would exceed that of lean control fibroblasts remains to be seen.

Although this *in vitro* study demonstrated no difference in TGF-β1 concentration in the conditioned medium of lean and obese JCR rat fibroblasts, we did observe a higher concentration of TGF-β1 in day 21 wounds of our *in vivo* study. *In vivo* other cells may be present which also produce TGF-β1, such as macrophages. Wetzler *et al.* (23) found that in db/db mice, the inflammatory phase of healing was prolonged as evidenced by the expression of the neutrophil chemoattractant macrophage inflammatory protein-2 (MIP-2) and the monocyte chemoattractant protein macrophage chemoattractant protein-1 (MCP-1). Both chemokines were expressed until the late phase of repair, resulting in high numbers of neutrophils and macrophages in late wound tissues.

In our *in vitro* model, many factors involved in wound healing were not included. The growth factors released from other cells such as adipocytes, inflammatory cells and

platelets serve to stimulate fibroblasts to migrate, proliferate, and synthesize growth factors and ECM proteins. Although we did not see a significant difference in the fibroblast proliferation in this study, there was a trend indicating a reduced proliferation of fibroblasts from obese JCR rats compared to lean. We used three strains from each group in this study and a larger sample size may have yielded significant results. Alternatively, without the influence of the wound environment, the differences between the fibroblasts explanted from lean and obese JCR rats may not be apparent due to insufficient activation.

Another difference between our *in vivo* rat study and this *in vitro* fibroblast model was that, although we observed a significant decrease in the contraction of the obese rat wounds compared to lean rat wounds, we did not show a significant difference in the contraction of fibroblast-populated collagen lattices. Again, this difference may be due to some missing fibroblast activating factor in our *in vitro* system. It may also be that the physical stretching of the obese rat skin to accommodate excess adipose tissue prevents the wounds from contracting properly.

Due to a mutation in the leptin receptor in the obese JCR rat, there is no leptin signaling in these animals. The absence of adipose tissue in our *in vitro* model resulted in the lack of adipocyte–derived leptin in the system. This may be one factor which would have stimulated lean rat fibroblasts and resulted in a significant difference in the behaviour of lean JCR rat fibroblasts compared to obese JCR rat fibroblasts. Leptin has been shown to act as a mitogen for keratinocytes (2). The influence of leptin on

keratinocytes during reepithelialization was studied in *ob/ob* mice. It was demonstrated that leptin improved reepithelialization of excisional wounds and accelerated wound healing in *ob/ob* mice. Leptin-treated animals exhibited a highly organized epithelium whereas PBS-treated wounds were disorganized and atrophied. The involvement of defective leptin signaling on fibroblast behaviour during wound healing has not yet been studied extensively.

Fibroblasts and adipocytes share a common stem cell origin and fibroblasts may be able to differentiate into preadipocytes and mature adipocytes (24, 25). Glasow *et al.* (5) showed mRNA and protein for leptin full length receptor Ob-Rb and mRNA for 3 shorter isoforms in fibroblast cultures, and suggested that the leptin receptor may be important for local effects of leptin on fibroblast function such as mitogenesis, chemotaxis and cytokine production. The investigators showed that fibroblasts secreted leptin into supernatants and they measured the same concentration as cultured human adipocytes. The concentration was related to high cell density, and may therefore be higher in non-proliferating cells.

Chronic hyperinsulinemia has been shown to increase leptin secretion from fibroblasts. Treatment of fibroblast cultures with physiological concentrations of insulin (1 nmol/L) increased fibroblast secretion of leptin to 152% of basal levels (5). Glasow suggested that fibroblast-derived leptin may have local effects or serve as an endocrine pool, the local action of leptin may affect collagen and cytokine synthesis, which may contribute to wound repair (26, 27).

Our study involved the explantation of fibroblasts from lean and obese JCR rats so that their behavior could be studied *in* vitro in the absence of other confounding factors. The only statistically significant difference we observed in fibroblast behavior was a decrease in hydroxyproline concentration in the conditioned medium from the obese JCR rat fibroblasts, although we did note trends in fibroblast proliferation and fibroblast-populated collagen lattice contraction. The fibroblasts from obese rats were not as proliferative as lean rat fibroblasts and the lattices populated with obese rat fibroblasts did not contract as much as those populated with lean fibroblasts. There are several possible reasons why we did not observed significant differences in many of the above assays. First, there was no adipose tissue or adipokines present to affect fibroblast behavior. Second, that there was no insulin present to increase the production of leptin from lean JCR rat fibroblasts. It would be interesting to see if the growth-promoting features of insulin would have been as effective on obese JCR rat fibroblasts. And third, in wound healing, the most significant effects of leptin target keratinocyte and not fibroblast behavior.

Many different cells and growth factors are involved in wound healing. An *in vitro* situation is designed to simplify a situation in order to focus on a small number of variables. The disadvantage of simplification is to inadvertently omit factors which may be key features of the disorder. However, by starting with a simplified model, we are now able to add variables to study the effect they may have on fibroblast behavior. Some of these additional experiments include; co-culturing fibroblasts with adipose tissue

explants to demonstrate the involvement of adipokines on fibroblast behavior, incubating lean and obese fibroblasts with insulin to see if lean rat fibroblast activity is significantly different from obese rat fibroblasts due to an increase in leptin receptor and protein expression, and finally to assess the role of the keratinocytes from the obese JCR:LA-cp rat.

VII. FIGURES AND LEGENDS

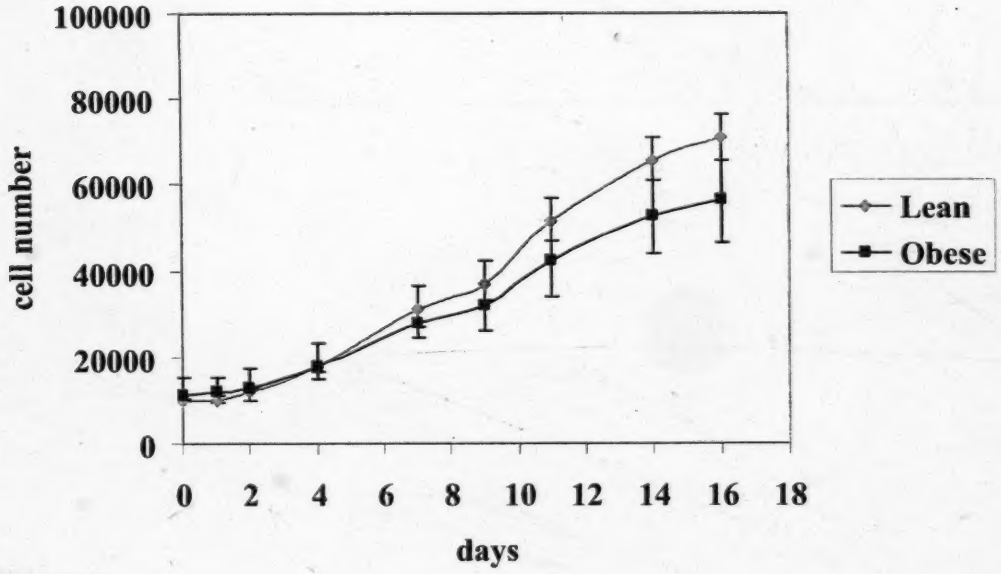


FIGURE 5-1

Cell counts for fibroblasts from lean and obese rats to day 16. There is a trend indicating lower proliferation of the fibroblasts from obese rats compared to lean but the trend did not reach statistical significance (lean 71008.15 ± 5396.11 vs. obese 56347.41 ± 10048.51 cells at day 16). n=3 strains for each group.

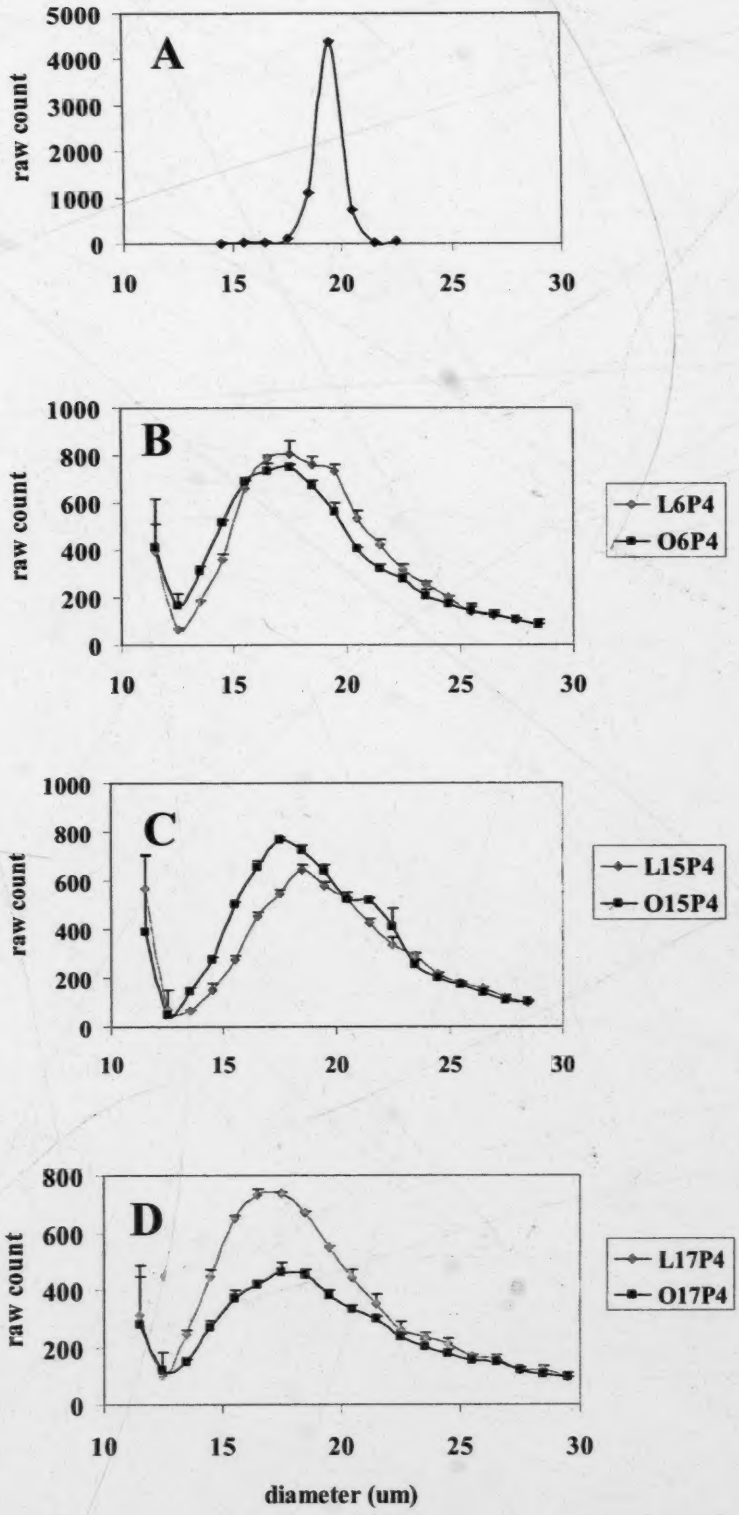

A
raw count
5000
4000
3000
2000
1000
0
10
15
20
25
30
B
raw count
1000
800
600
400
200
0
L6P4
O6P4
C
raw count
L15P4
O15P4
D
raw count
800
600
400
200
0
L17P4
O17P4
diameter (um)

FIGURE 5-2

Comparison of cell diameter. Coulter counter was gated from 10 to 30 μm. Latex bead calibration (21.5 um) (a). Three different strains of fibroblasts from lean and obese rats were measured (b, c, d). There were no obvious differences in cell size with the strains of fibroblasts analyzed. (O= obese rat fibroblast strain, L= lean rat fibroblast strain, P4 indicates passage number).

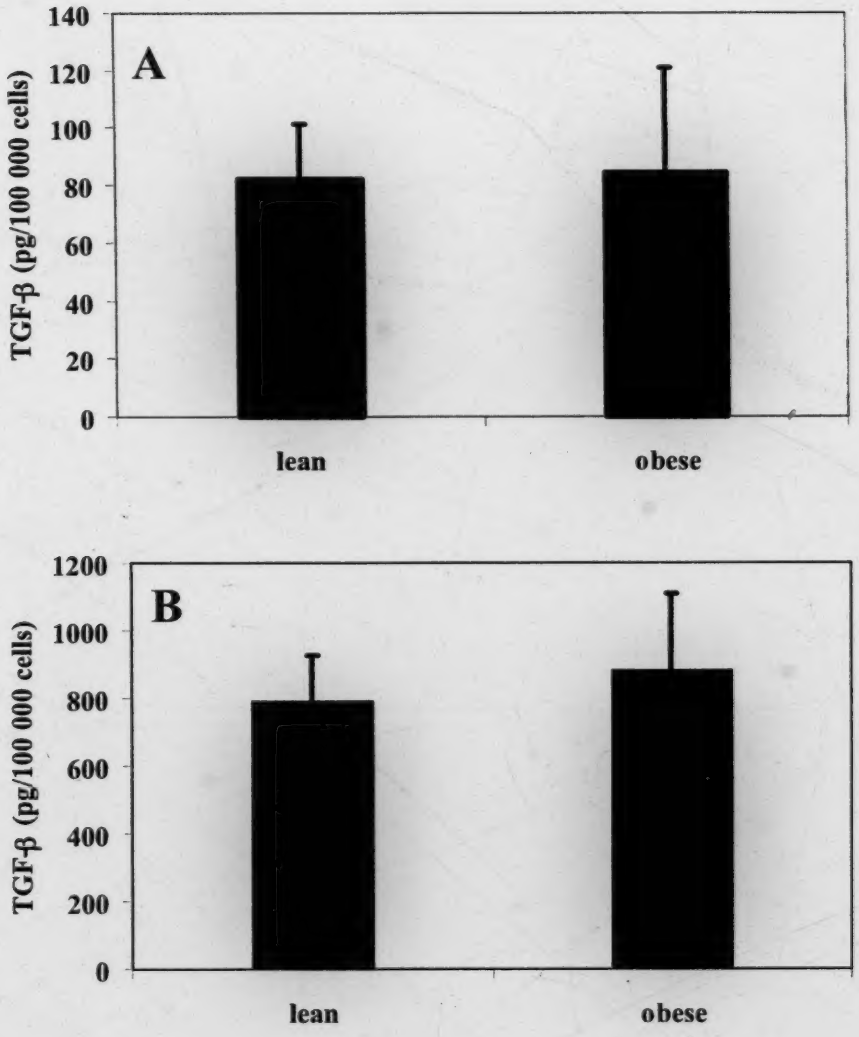
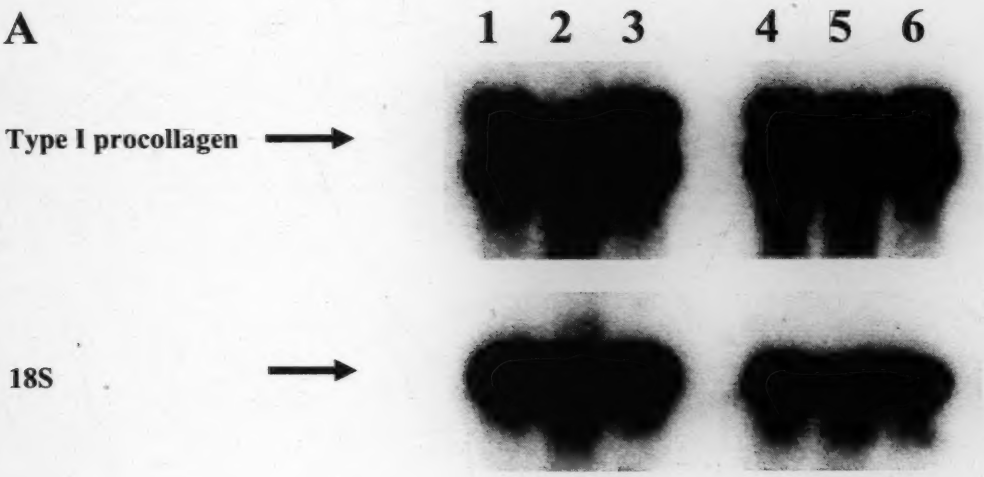


FIGURE 5-3

TGF-β ELISA. Active TGF-β1 (a) was measured using non-acidified conditioned medium samples and total TGF-β (b) was measured following acidification of conditioned medium. Two separate experiments, n=3 fibroblast strains for each experiment. There were no significant differences in active and total TGF-β1 levels between lean and obese JCR:LA-cp rat fibroblasts.

A


1 2 3 4 5 6
Type I procollagen
18S

B

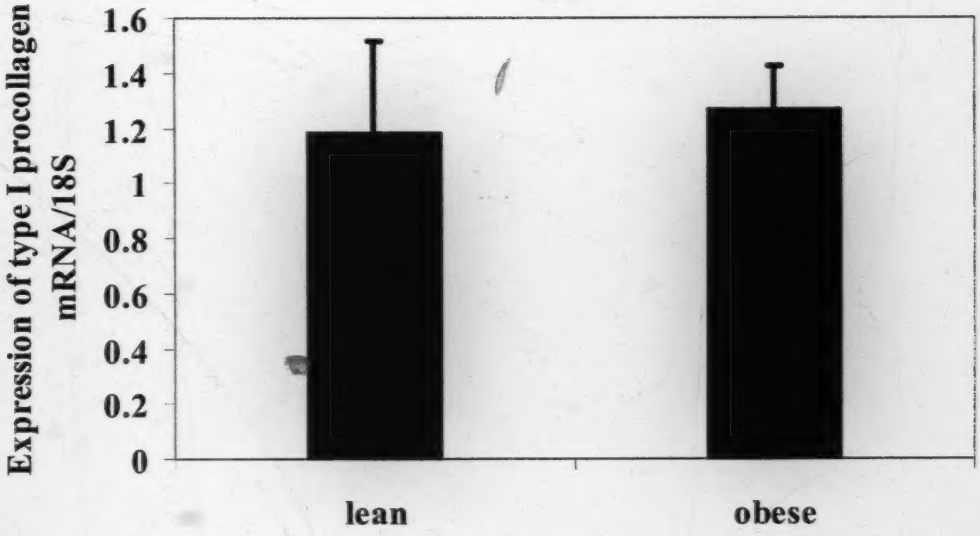

Expression of type I procollagen mRNA/18S
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
lean
obese

FIGURE 5-4

Northern analysis of type I procollagen (a) with 18S loading control. Lanes 1-3 are 3 strains of lean rat fibroblasts, lanes 4-6 are 3 strains of obese rat fibroblasts. Relative expression of type I procollagen following normalization with 18 S (b). Blot is representative of two separate experiments each with 3 strains of fibroblasts per group. Following normalization with 18S rRNA, no significant differences were observed in type I procollagen expression between lean and obese JCR:LA-cp fibroblasts.

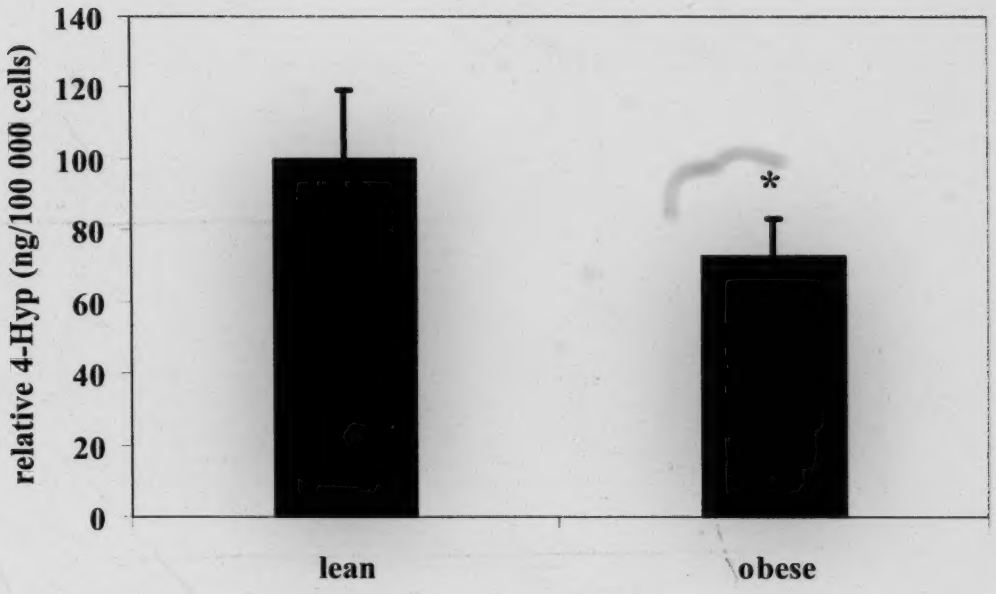


FIGURE 5-5

Relative hydroxyproline measurement from conditioned medium. Two separate experiments, 3 strains of fibroblasts for each group. Normalization between assays was achieved by multiplying the mean of the lean rat fibroblast results by a factor to equal 100 and using the same factor for obese rat fibroblast results. *P=0.0118.

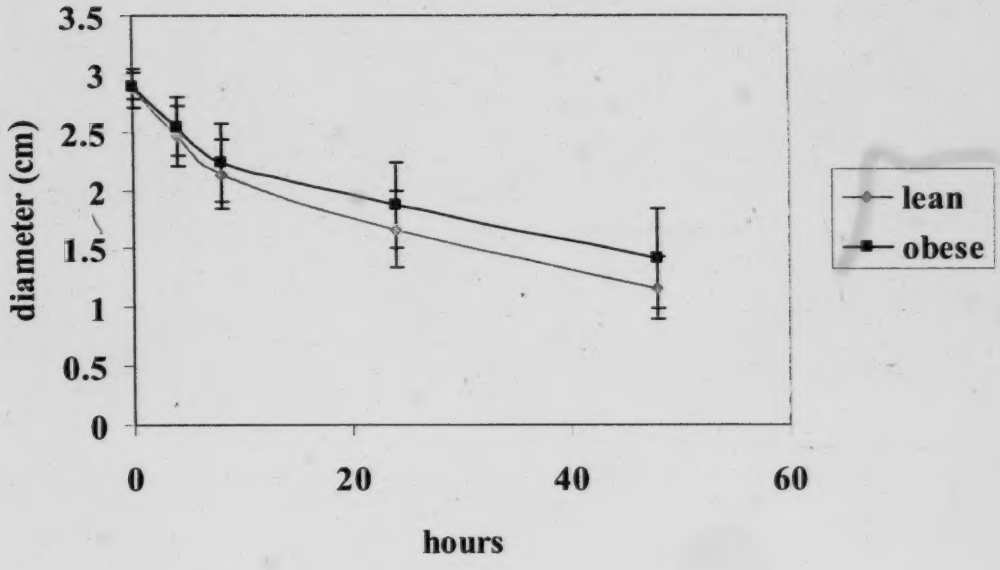


FIGURE 5-6

Fibroblast-populated collagen lattice contraction. Gels were released and allowed to contract over 48 hours. Measurements of the gels were taken at the time of release (0 hours), and at 4, 8, 24 and 48 hours. Two separate experiments, 2 strains of fibroblasts for each group. A trend was noted in which gels populated with obese JCR:LA-cp rat fibroblasts contracted less than those populated with lean rat fibroblasts, but statistical significance was not achieved.

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CHAPTER 6

Healing of the JCR:LA-cp Rat Following MEDICA16 and S15261 Treatment

I. ABSTRACT

Due to a genetic defect resulting in loss of leptin receptor function, the obese JCR:LA-cp rat has characteristics similar to those seen in humans with metabolic syndrome. The male homozygous rats are obese, hyperinsulinemic, hypertriglyceridemic, and are prone to atherosclerosis. We have previously shown that male JCR:LA-cp/cp rats have impaired wound healing compared to lean controls and sought to improve healing by treating the underlying condition with either MEDICA 16 or S15261. MEDICA 16 decreases VLDL hyperlipidemia and insulin levels and is cardioprotective and antiatherosclerotic. S15261 decreases body weight and insulin levels and has been developed to treat metabolic syndrome and type 2 diabetes in humans.

We treated obese JCR rats with either MEDICA 16 or S15261 for four weeks from age 8 to 12 weeks and then performed 6 excisional biopsy wounds on each treated group, untreated obese JCR rats, and their age-matched lean controls (8 rats per group). Animals were weighed on a weekly basis throughout the study and plasma was collected for insulin measurement. Wound edges were traced during a 14 day period to assess wound contraction. On day 14 wounds were collected and hydroxyproline content was measured in unwounded tissue and day 14 wounds.

We found that insulin levels were significantly lower with both MEDICA 16 and S15261. Although body weights were significantly lower than obese controls with S15261 starting at day 11 and with MEDICA 16 at day 13, they were still significantly

higher than lean controls. We did not find a significant improvement in wound contraction or hydroxyproline content with either drug. However, changes in protocol such as a longer drug treatment regimen beginning at an earlier age may result in significant improvements.

II. RATIONALE FOR CURRENT STUDY

The initial characterization of the JCR:LA-cp rat has demonstrated a decrease in wound contraction and hydroxyproline content in the day 21 wounds of the obese rats, and a decrease in the hydroxyproline production *in vitro* by fibroblasts explanted from obese rats. Further characterization of this model is required both *in vitro* and *in vivo.* Some studies might include the study of other growth factors involved in wound healing such as IGF-1 and VEGF, metalloproteinases, and whether there is a delay in inflammatory cell migration into the wound.

We are also very interested in knowing whether altering the metabolic state of the obese JCR:LA-cp rats would be sufficient in overcoming the impaired healing observed *in vivo*. One approach is to treat these rats with drugs known to lower insulin and triglyceride levels and decrease weight gain. Two drugs that have been shown effective in this model are MEDICA 16 and S15261. We propose to use each of these drugs to demonstrate if normalizing some of the metabolic abnormalities in these rats will have a positive effect on wound healing.

III. INTRODUCTION

We recently demonstrated impaired healing of full thickness wounds in the JCR:LA-cp/cp rat. The next goal was to test the effect on healing of these rats with two drugs shown to decrease body weights and plasma insulin levels of JCR:LA-cp/cp rats (courtesy of Dr. Russell, University of Alberta, Canada). Since the defect in these animals is genetic (defective leptin receptors), rather than preventing insulin resistance, our approach was to treat the hyperinsulinemia and obesity in an effort to improve wound healing. We hypothesized that if impaired healing is due to the metabolic syndrome experienced by these animals, lowering insulin and body mass may have a positive effect on downstream factors such as wound healing.

MEDICA 16 (β,β'-tetramethylhexadecanedioic acid) is an inhibitor of citrate lyase, and important enzyme in triacylgycerol synthesis (1,2). Citrate lyase is an enzyme involved in the first step of triacylglycerol synthesis, the citrate transport system which transports acetyl CoA from the mitochondria to the cytosol. MEDICA 16 also decreases acetyl CoA carboxylase in the liver (3). Acetyl CoA carboxylase is a key regulator in the second step of triacylglycerol synthesis in that it converts bicarbonate and acetyl CoA into malonyl CoA in the cytosol. One function of malonyl CoA is to prevent the transport of long-chain fatty acids into the mitochondria for oxidation. In the JCR:LA-cp/cp rat, MEDICA 16 has been shown to decrease VLDL hyperlipidemia (2) and to reduce insulin levels, and is both cardioprotective and antiatherosclerotic (4).

S15261 (L-isomer of 2-({2-methoxy-2-[3-(trifluoromethyl)phenyl]ethyl}amino)

ethyl-4(2-{[2-(9*H*-9-fluorylenyl)acetyl]amino}ethyl)benzene) was developed for the treatment of metabolic syndrome and type 2 diabetes. Metabolic syndrome is characterized by abdominal obesity, hypertension and hypertriglyceridemia which usually precede atherosclerosis (5). The involvement of hyperinsulinemia in the development of cardiovascular disease is now recognized. Although the mechanism of action has not been determined, treatment with S15261 has previously been shown to decrease food intake and body weight, decrease plasma insulin levels, decrease the contractile response of mesenteric resistance vessels to norepinephrine, and increase the maximal nitric oxide-mediated relaxation (6). S15261 treatment has also resulted in a decrease in the mRNA of enzymes involved in gluconeogenesis and increases in the mRNA for enzymes of hepatic glucose utilization (6).

We have shown that JCR:LA-cp/cp rats have impaired healing compared to lean controls by demonstrating a decrease in wound contraction and decreased hydroxyproline content (7). Our goal in this study is to improve healing using treatments known to decrease the hyperinsulinemia and weight gain associated with the obese JCR rat.

IV. MATERIALS AND METHODS

A. Animals

Male rats of the JCR:LA-cp strain, obese (cp/cp) and lean (cp/+ or +/+), were raised in an established breeding colony at the University of Alberta as previously described (4). The rats were weaned at 3 weeks of age and housed initially in pairs in polycarbonate cages on woodchip bedding at 20°C and 55% relative humidity. The

lighting was on a 12-hour cycle. At 6 weeks of age the rats were housed individually. Food was available at all times (Teklad Rodent Diet; Harlan Sprague-Dawley, Madison, WI). This is a corn and wheat-based diet of less than 4% total lipid and 23% protein content; the energy content is approximately 3.3 kcal/g. Drugs were administered from 8 to 12 weeks of age. The groups consisted of 8 lean controls, 8 obese controls, 8 obese Medica treated (0.25%w/w) and 8 obese S15261 treated (37.2 mg/kg/day). Rats were wounded at 12 weeks and followed for 14 days at which time they were terminated and wounds were measured and collected.

B. Plasma Measurements

Plasma insulin was measured by immunoassay (8).

C. Wound Generation

The rats were premedicated with buprenorphine 0.03mg/kg (Buprenex™, Reckitt & Coleman, Richmond VA. USA), and glycopyrulate™ 0.1mg/kg (Sabex Inc. Boucherville, Que., Canada). Surgical plane anesthesia was induced and maintained by facemask using isoflurane™ (Biomeda-MTC, Cambridge, ON) and O_2 to effect. The dorsal surface was washed with 70% ethanol and 10% Betadine™, (Purdue Fredrick Inc. Toronto, Ont., Canada). Six full thickness excisional wounds were made on each rat (3 wounds on each side of the midline) using a 6 mm Accu Punch™ disposable biopsy tool (Dormer Laboratories, Mississauga, Ont. Canada). There was no experimental intervention in the wounds and six animals from each experimental group were sacrificed at day 14. Wound tracings were made on transparency film to assess wound contraction.

Transparencies were scanned and planimetry was performed using Scion Image (NIH, USA) with a scale on each transparency for calibration. For the initial surgery (day 0) and at termination, samples were collected for hydroxyproline content and were snap frozen and stored at –80°C.

D. Hydroxyproline Analysis

The content of collagen in unwounded and wounded tissue was determined by mass spectrometric analysis for 4-hydroxyproline (9). Skin samples from lean and obese rats were freeze-dried. Internal standard (*N*-methyl-L-proline) and 6 N HCl solution was added to wound tissue, and each sample was then hydrolyzed overnight at 115 °C. The *O*-butyl ester derivatives were prepared with 10% BF_2-butanol for 30 min at 120 °C after drying the hydrolysate. Liquid chromatography (using a Eclipse XDB-C18 column)/mass spectrometry analysis was performed on a Hewlett-Packard (series 1100, Atlanta, GA) mass selective detector monitoring the ions at m/z 188 and 186.

E. Statistical Analysis

A one way ANOVA was used to analyze groups (Tukey-Kramer Multiple Comparison) using GraphPad InStat 3 (San Digeo, USA). Each group consisted of eight animals. A $P<0.05$ was considered statistically significant.

V. RESULTS

Animals were weighed on a weekly basis (fig 6-1). At all time points, the weights of the JCR:LA-cp obese controls as well as both drug treatment groups were significantly

higher than the lean controls (P<0.001). However, the weights in both drug groups did lower significantly compared to the obese controls. Starting at week 11, the S15261 group weighed significantly less than the obese control (P<0.05; S15261 406.63±20.13 vs. obese 440.75±19.13) and this difference was most significant at week 13 (P<0.001; S15261 446.38±17.92 vs. obese 494.75±12.74). At week 13 the MEDICA 16 group weighed significantly less than the obese controls (P<0.01, MEDICA 16 456.00±26.24 vs. obese 494.75±12.74) after which the difference was no longer significant. As there was only one significantly different timepoint with MEDICA 16, it is possible that this is an outlier.

Plasma samples were obtained at termination and insulin levels were measured. The insulin levels in the plasma of the obese control were significantly higher than in the lean control (P<0.0001, obese 346.80±127.72 vs. lean 97.68±46.04) (fig 6-2). The insulin levels for both drug treatment groups were lowered to levels not significantly different from the lean control. Each treatment group was significantly lower than the obese control (P<0.01, MEDICA 16 178.24±73.41, S15261 191.41±80.30 vs. obese 346.80±127.72).

Figures 6-3 to 6-5 represent wounds at day 0, 1, and 14 respectively. At the time of wounding, the wound tracings showed no differences in contraction between groups (fig 6-6). However, at day 1 post-wounding, there was a significant difference in contraction between the lean controls and all other groups (P<0.01; lean 1.18±0.12, obese 1.40±0.10, MEDICA 16 1.41±0.08, S15261 1.40±0.12). As shown in our previous study,

the lean group contracts sooner than obese rats. By day 14 the difference between the lean control group, the obese controls and both drug treatment groups was still significantly different ($P<0.001$; lean 0.62±0.05, obese 0.98±0.13, MEDICA 16 0.93±0.05, S15261 1.01±0.09). Reepithelialization was not evaluated in this study as the wounds are usually scabbed over after day 1 and removing the scabs disrupts the wound beds.

Hydroxyproline levels were measured as an indication of collagen content. We found that both the obese control group and the MEDICA 16 treated groups had significantly less hydroxyproline than the lean control group ($P<0.001$) in both unwounded skin (obese 271.73±16.22, MEDICA 16 281.47±34.37, lean 369.30±20.11 μg/sample) and in day 14 wounds (obese 174.83±25.42, MEDICA 16 192.61±35.69, lean 322.39±24.28) (fig 6-7). Although the S15261 treated group also had significantly less hydroxyproline than the lean control ($P<0.01$) in unwounded skin (S15261 287.37±17.80 vs lean 369.30±20.11) and in day 14 wounds (S15261 222.54±32.65 vs lean 322.39±24.28), the level was slightly higher than both the obese control and MEDICA 16 treated groups.

VI. DISCUSSION

There are at least three possible reasons why there was no significant improvement in healing following drug treatment. The first reason being that neither MEDICA16 nor S15261, were sufficient in overcoming the genetic defect in these rats. The leptin receptor is defective in these rats, and although the drugs were able to reduce

insulin levels and had the potential to reduce obesity, that may not be sufficient to reverse the wound healing defect(s) in this model.

The second explanation may be that the drug treatment was not started early enough. Russell et al. (10) had shown that obese JCR rats treated with MEDICA 16 from age 6 weeks showed a significant reduction in plasma lipids but only a modest reduction in body weight and insulin resistance. However, when treatment was administered at 3 weeks at the time of weaning, weight was normalized for a longer period of time, insulin levels were reduced, and plasma triacylglycerol levels were the same as lean controls. Also, muscle triacylglycerol or fat droplets were not seen in the muscle tissue as they were in untreated obese rats. This study concluded that the significantly elevated triacylglycerol levels observed at 3 weeks preceded insulin resistance and hyperinsulinemia.

The results with S15261 treatment did look more promising than with MEDICA 16. The weights of the obese rats were significantly less than the obese controls beginning at week 11 and the hydroxyproline content was slightly greater than both the obese controls and the MEDICA 16 treated group. However, the slight improvements observed with S15261 did not significantly improve the healing in obese rats when compared to the lean controls.

Finally, the healing impairment in these rats may be primarily due to the underlying fat mass splinting the wounds open. The changes in leptin signaling may be a

minor factor in this model. Perhaps a longer treatment with the drugs may have mediated a significant weight loss and this alone would be sufficient for improving healing. With less adipose tissue, the blood supply to the wound would be improved (11). Additionally, excess adipose tissue where moisture is able to accumulate has been shown to increase bacterial growth and promote wound infection (12-15). Although we didn't observe wound infection in our model, it is a very important factor in the healing of obese humans and treatment targeting insulin resistance and weight normalization may be key.

VII. FIGURES AND LEGENDS

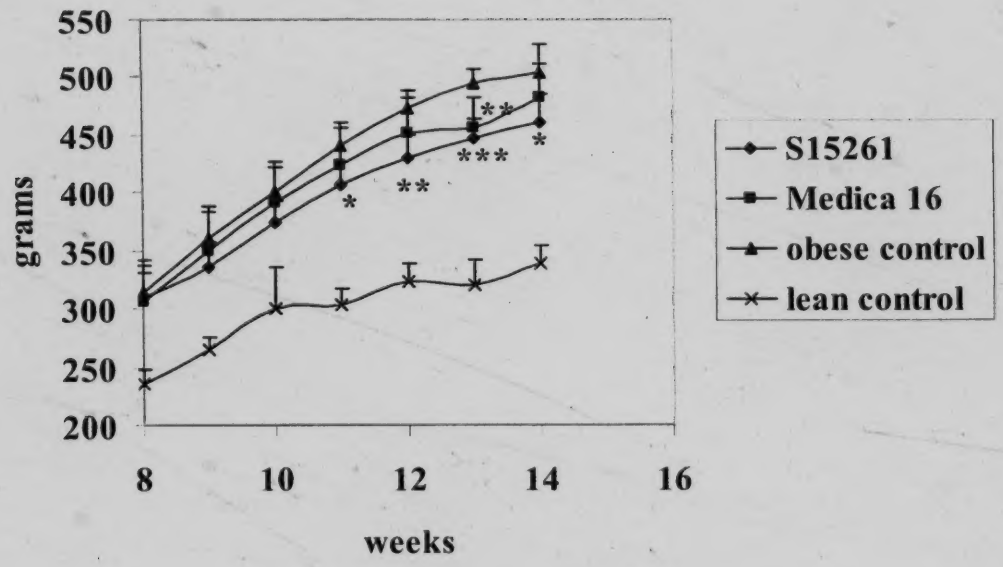

FIGURE 6-1

Rat body weights in grams from week 8 to week 14. The weights of obese rats treated with S15261 were significantly less than the obese control beginning at week 11 and remained significantly lower for the duration of the study. Obese rats treated with Medica 16 had significantly lower body weights than the obese controls at week 13 only. Neither drug treatment lowered weights to lean control levels. *P<0.05, **P<0.01, ***P<0.0001 (n=8 per group).

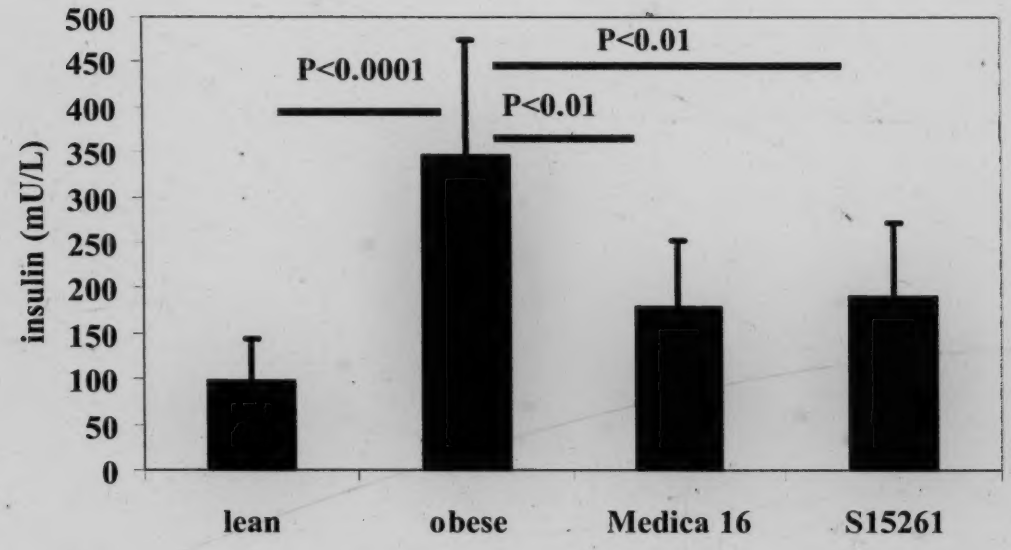

FIGURE 6-2

Plasma samples were obtained at study termination for insulin measurement. Both Medica 16 and S15261 significantly lowered insulin levels to that of the lean control. The obese rat control remained significantly higher than the lean control and both drug treatment groups (n=8 per group).

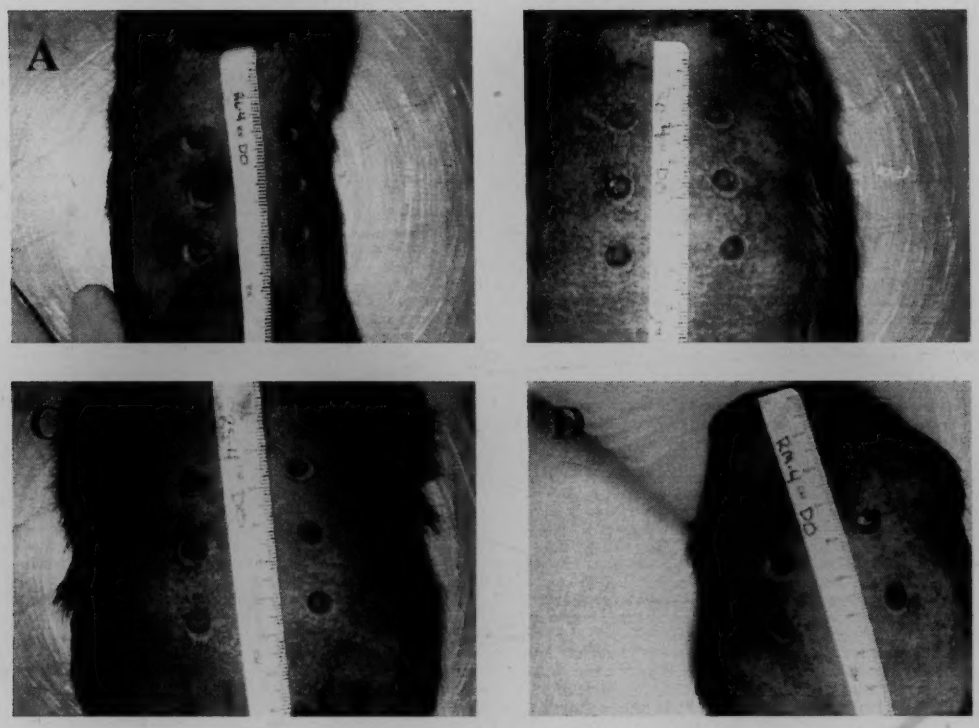

FIGURE 6-3

Representative wound appearance at Day 0. Six excisional wounds were made on the dorsal surface of each rat with a 6 mm punch biopsy tool. Lean control (a), obese control (b), S15261 treated (c), and MEDICA 16 treated (d). At the time of wounding there were no obvious differences in the appearance of the wounds.

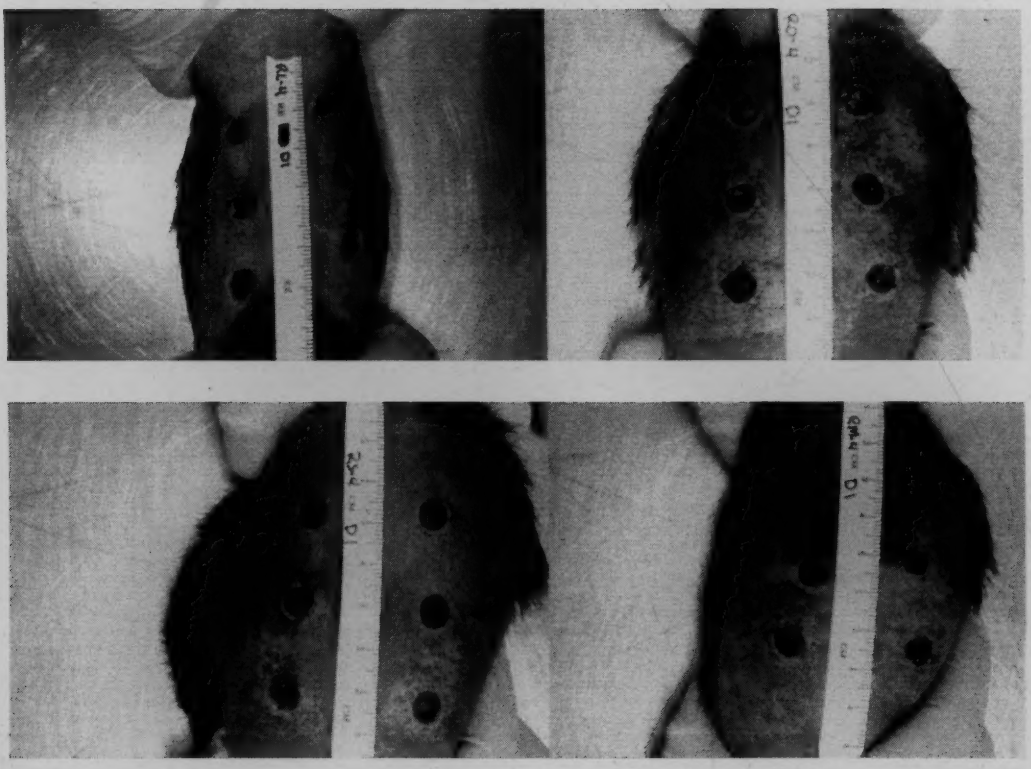

FIGURE 6-4

Representative wound appearance at Day 1. Lean control (a), obese control (b), S15261 treated (c), and MEDICA 16 treated (d). At day 1 post-wounding, there appeared to be slightly more wound contraction in the lean control group compared to the obese control and both drug treatment groups.

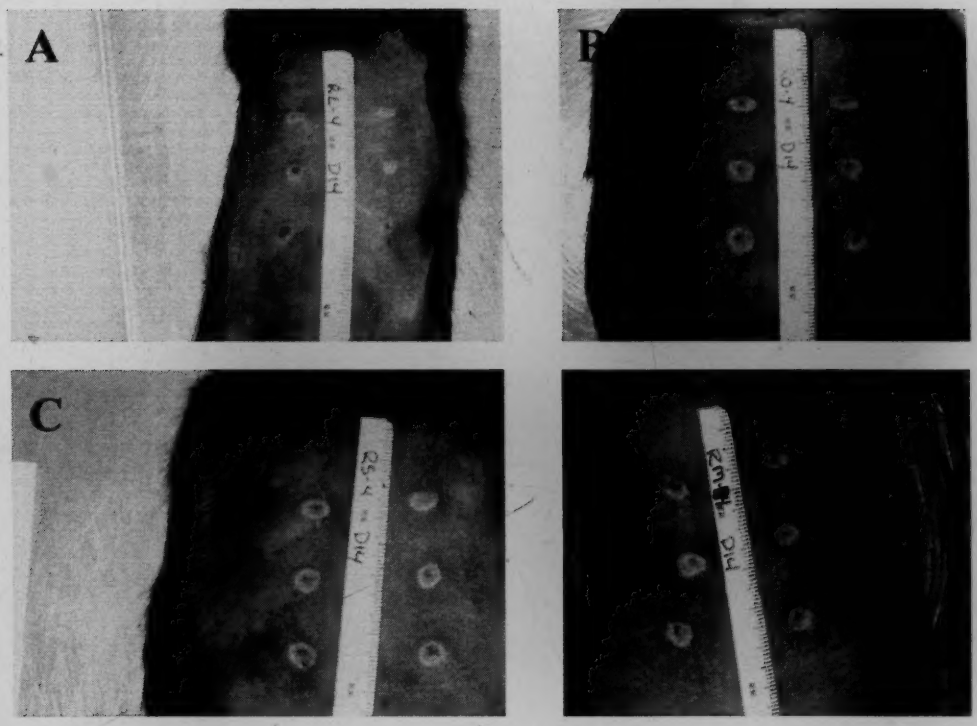

FIGURE 6-5

Representative wound appearance at Day 14. Lean control (a), obese control (b), S15261 treated (c), and MEDICA 16 treated (d). At day 14 post-wounding the differences in wound contraction were visually apparent. The lean control rat wounds contracted more than the obese control and both drug treatment groups.

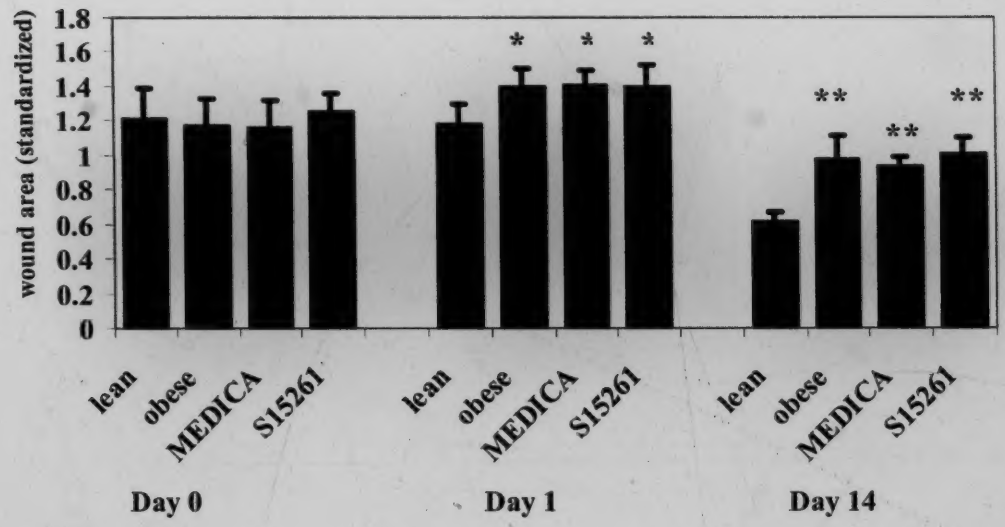

Figure 6-6

Wound contraction following standardization. At the time of wounding (day 0), there were no apparent differences in wound appearance. Significantly more contraction was noted in lean control rats at day 1 post-wounding compared to obese controls and both drug treatment groups. At day 14, the difference between the lean control group and all other groups was most significant. *P<0.01, **P<0.001 (n=8 per group).

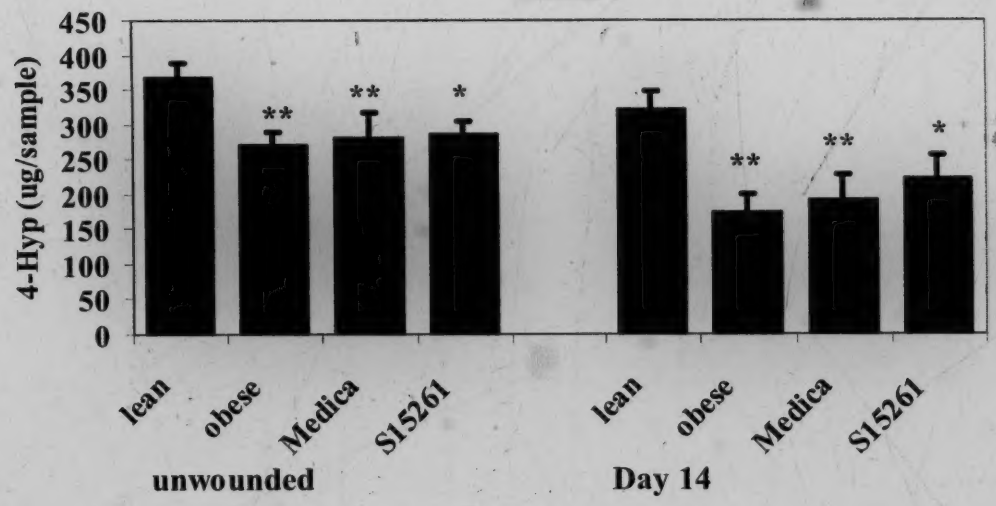

FIGURE 6-7

Hydroxyproline levels in 6 mm punch biopsies of unwounded skin and day 14 wounds. For both unwounded tissue and tissue collected at day 14, the obese controls and both drug treatment group wounds had significantly lower hydroxyproline content than the lean controls. The hydroxyproline content of the skin samples collected from the S15261 treatment group was slightly greater than the obese control group and the Medica 16 treated group and was not significantly different from either. *P<0.01, **P<0.001 (n=4 per group).

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CHAPTER 7

Conclusions

I. SUMMARY

Through this series of experiments we have; 1) successfully shown that an adenovirus vector containing active or latent TGF-β1 cDNA can be used to transduce human keratinocytes, 2) begun the characterization of the JCR:LA-cp rat, a novel model for impaired healing, 3) demonstrated that the primary deficiency of healing impairment of the JCR:LA-cp rat is probably upstream of fibroblast involvement as the *in vitro* behavior of lean and obese JCR rat fibroblasts does not appear to be significantly different apart from there being less hydroxyproline production from the obese rat fibroblasts, and 4) that treatment of JCR:LA-cp rats with MEDICA 16 or S15261 did lower insulin levels but was not successful in resolving wound healing deficiencies.

Our first experiments involved transducing human keratinocytes with active and latent TGF-β1. In co-culture with human fibroblasts, these transduced keratinocytes were capable of modulating fibroblast behavior. We demonstrated that the TGF-β1 produced by the keratinocytes was capable of traversing the membrane separating the two cell types in our *in vitro* model. Furthermore, the levels of latent and active TGF-β1 were significantly higher in the respective transfected keratinocytes than in those transduced with the LacZ control virus. The TGF-β1 produced by the transduced keratinocytes was shown to be biologically functional using the plasminogen activator inhibitor (PAI/L) assay, and both latent and active TGF-β1 induced the fibroblasts to proliferate significantly more than the LacZ control. To demonstrate that the TGF-β1 had an effect on the underlying fibroblasts, cell proliferation was assessed and mRNA was extracted and northern analysis was performed. We found that latent TGF-β1 significantly

increased the expression of type I collagen mRNA but did not significantly affect collagenase mRNA. However, active TGF-β1 significantly increased type 1 collagen mRNA while also significantly decreasing collagenase activity.

We then attempted to take our *in vitro* model of gene therapy to an *in vivo* model. We sought a larger rodent model as mouse skin is too delicate to inject with the volumes of virus we required. Through Dr. James Russell, we were introduced to the JCR:LA-cp rat. The JCR rat is a model of insulin resistance that has been used extensively to study atherosclerosis but until now, has not been used to study wound healing.

Unfortunately we were unable to successfully transduce the surrounding cells of the wounds with the adenovirus. Adenovirus invades target cells by first binding to the Coxsackievirus and adenovirus receptor (CAR) on the cell membrane. CAR expression is low or absent in human fibroblasts (1). Consequently these cells are difficult to infect with Ad5-based vectors (2). The adenoviral vectors used in our study contained the left end of human adenovirus type 5 genome (3). Our initial study with the recombinant adenoviruses involved transducing human keratinocytes *in vitro* and we were successful in showing the expression of the latent and active TGF-β1 transgenes and some biological effects. However, in our *in vivo* model, due to the relative size of our syringe compared to the epidermis we injected most of the adenoviruses into the dermis. We were unable to show successful transduction in the skin, the main reason being that fibroblasts are the primary cell type of the dermis. Due to the complexity of gene

therapy, we decided to change our focus instead to the characterization to the JCR:LA-cp rat as a novel healing impaired model.

We had noted obvious differences in the healing of the obese JCR rats compared to the lean controls in our preliminary work and decided to focus on developing a wounding protocol for these animals. The *db/db* mouse is used extensively for wounding studies and a rat model with a similar genetic defect may prove valuable in the research community. We created six full thickness excisional wounds on the dorsum of the obese rats and their lean controls. Tracings were taken to assess rate of wound closure and although we did not observe a difference in reepithelialization, we did note a decrease in wound contraction in the obese rats. We also found a decrease in hydroxyproline content in the obese wounds at day 21 post-wounding which may be due in part to an increase in adipose tissue in place of dermal tissue, as noted in the histology of the skin. Based on these results, we believe that the JCR:LA-cp rat is a suitable model for healing impairment, the factors causing this impairment and possible treatments to improve healing should be pursued. Our deficiency in wound contraction and low hydroxyproline results in the obese rats suggested a defect in fibroblast behaviour. Therefore, skin samples were obtained for lean and obese rats and fibroblasts were explanted.

We performed several *in vitro* analyses using fibroblasts from lean and obese JCR rats including; cell proliferation, cell size, TGF-β1 ELISA from conditioned medium, northern analysis of type I procollagen mRNA, hydroxyproline concentration in conditioned medium, and fibroblast-populated collagen lattice contraction. We failed to

find significant differences in any of the experiments other than hydroxyproline concentration in the conditioned medium. We found that the fibroblasts explanted from obese JCR rats produced significantly less hydroxyproline that the lean controls (P=0.0118, obese 72.59±10.70 vs. lean 100.00± 10.70). As fibroblasts are an essential cell in the production of collagen during healing, this is an important finding.

It was surprising that we did not find any other significant differences between lean and obese rat fibroblasts. This may be due to the small sample sizes. Each experiment required very high cell densities and therefore, included only three strains each of lean and obese fibroblasts, although the experiments were all performed in duplicate. There are other possible explanations for the similarities in fibroblast behaviour. The first being that we were studying only one cell type from a very complex wound environment. It may be that in the absence of adipose tissue, many of the factors which influence fibroblasts are absent. Adipose tissue is now considered to be an important endocrine tissue capable of producing several proteins (adipokines) which have been shown to travel to various target tissues and regulate other cell functions. A second reason for a lack of difference in lean and obese fibroblast behaviour in most of our *in vitro* assays may be the lack of insulin in the system. Glasow *et al.* (4) demonstrated that insulin is able to stimulate fibroblast synthesis and secretion of leptin and also, fibroblasts express all of the leptin receptor isoforms. Although the fibroblasts from the obese rats would be capable of producing leptin in the presence of insulin, they would not be capable of responding to leptin in an autocrine manner due to a mutation in the leptin

receptor. Glasow suggested that the local actions of leptin may affect collagen synthesis and cytokine release which may then improve wound repair.

We sought to determine whether the healing deficiencies seen in obese JCR rats could be prevented by treating the rats with MEDICA 16 or S15261, drugs known to correct hyperinsulinemia. We found that although these two drugs did significantly lower insulin levels, they did not improve wound contraction or increase hydroxyproline content to that of the lean control. S15261 was slightly more effective in lowering the JCR:LA-cp body weight compared to the obese control and the hydroxyproline content of wounds from animals treated with S15261 was slightly greater than the obese control. However, the results following treatment with S15261 were still significantly different from the lean control. It may be that by starting drug treatment at weaning (3 weeks old), the obese rats would have shown a significant improvement in healing as they would not have gained as much weight and therefore the underlying adipose would have been substantially less. This would benefit healing because the levels of adipokines would have been closer to normal levels. The dermis may also be stronger due to decreased infiltration of adipose tissue. We may also have demonstrated increased contraction of obese rat wounds if the physical presence of the adipose tissue was causing the wounds to be splinted open.

II. SUGGESTIONS FOR FUTURE EXPERIMENTS

A. Further Characterization of the JCR:LA-cp rat

There is still much to learn about the healing of the JCR:LA-cp rat including growth factor expression, the length of the inflammatory phase, and metalloproteinase expression. There are many advantages to using rats rather than mice in skin research including the increased thickness of the skin and the larger size of the animal allows for more wounds of a larger size to be created. Several studies have been performed using the *db/db* mouse which is also leptin resistant due to a leptin receptor mutation. Many of these experiments should be performed in the JCR rat to see if this model is as valuable to those studying wound healing. Although the JCR:LA-cp rat and the *db/db* mouse both have mutated leptin receptors resulting in defective leptin signaling, the genetic defects are on different species which may cause dissimilar outcomes in some aspects of healing.

Thus far, the only growth factor we have measured in the skin of the JCR:LA-cp rat is TGF-β1 and we found no significant differences in serum or tissue TGF-β1 levels between genotypes. In the *db/db* mouse, many growth factors have been studied and been found to be altered. These include VEGF (5, 6, 7), PDGF (8), IGF-I and IGF-II (9). It has also been shown that the inflammatory phase of healing is prolonged in *db/db* mice (10). And finally, the expression of metalloproteinases is altered in *db/db* mice much as it is in chronic wounds in humans (11). In our *in vivo* study with the JCR:LA-cp rats, we measured a decrease in hydroxyproline content at day 21 in the obese rats. One

explanation may be the breakdown of collagen by increased metalloproteinases in the obese animals.

We would propose to use our rat wounding protocol for lean and obese JCR:LA-cp rats and follow wounds for up to 4 weeks. Rats would be terminated each week for the following assays. Either immuonhistochemistry or northern analysis will be used to measure VEGF, PDGF, IGF-I and IGF-II expression in both unwounded tissue and in wounds from lean and obese rats. The prolongation of the inflammatory phase would be assessed by measuring TNF-α mRNA, a pro-inflammatory cytokine, and performing immunohistochemistry to measure PMN- and macrophage-specific antigen (10). Gelatin zymography would be used to measure MMP-2 and MMP-9 (11).

B. Fibroblast and Adipose Tissue Co-culture

Our lab has had limited success in the culture of adipocytes from tissue explants due to their short life-span. Brunn *et al.* (12) was able to use adipose tissue biopsies in organ culture for up to 72 hours. We propose to use a similar system as in our TGF-β1 transduction study in which an insert was placed on top of one cell type with a second cell type growing on the insert itself. In this case, we would place the insert on top of the adipose tissue biopsies to prevent them from floating and fibroblasts would be cultured on the porous membrane of the insert. Adipokines from the tissue biopsies and cytokines from the fibroblasts would then be capable to transversing the membrane to influence cells in the other compartment. Various assays would be performed on both the

fibroblasts and supernatants after the incubation period. Conditioned media would be collected to measure ECM protein and cytokine levels: hydroxyproline, TGF-β1 (ELISA (13) or PAI/L bioassay (14) depending on required sensitivity), and also adipokine levels of leptin (RIA (4) or luciferase bioassay (15)), adiponectin (ELISA (16)), and TNF-α (ELISA (17), or bioassay (18)). Two-dimensional electrophoresis could also be performed on fibroblasts and supernatants to determine the influence of the adipose tissue on levels of specific cell proteins.

Various conditions of the above co-culture system would be studied. In the first, both adipose tissue and fibroblasts from obese JCR rats would be co-cultured (FO/AO) and compared to adipose tissue co-cultured with fibroblasts from lean rats (FL/AL). These results would be compared to control fibroblasts from each rat strain cultured in the absence of adipose tissue. In this set of experiments the lean co-culture (FL/AL) results would serve as a baseline, the obese co-culture (FO/AO) would give some indication as to whether the fibroblasts are not responding as well to the signals from the adipose tissue due to a lack of functional leptin receptors.

Another set of experiments would include adipose tissue from obese rats co-cultured with fibroblasts from lean rats (FL/AO) compared to adipose tissue from lean rats co-cultured with fibroblasts from obese rats (FO/AL). The FL/AO result would indicate whether the differences in fibroblast behavior are due to aberrant adipokine release from the adipose tissue derived from obese rats, or if there isn't a significant difference between this condition and FL/AL, the defect may be primarily due to

fibroblasts from obese rats which leads to the last co-culture consisting of fibroblasts from obese rats co-cultured with adipose tissue from lean rats (FO/AL). If fibroblast behavior is influenced greatly by leptin signaling from adipose tissue (and also autocrine signals from fibroblast-derived leptin), this co-culture system will demonstrate that in the presence of normal adipose tissue, fibroblasts fail to be stimulated as fibroblasts from lean rats would.

C. Insulin Treatment of Fibroblasts from JCR:LA-cp Rats

It has been shown that aortic smooth muscle cell (SMC) proliferation *in vitro* was positively correlated with plasma insulin levels *in vivo* and aortic SMCs from JCR:LA-cp rats treated with insulin showed an increase in proliferation (19). The aortic SMCs in the obese JCR:LA-cp rat are thought to contribute to atherosclerosis due to their neointimal migration, proliferation and ECM production (20, 21). However, our proliferation experiments with fibroblasts from obese JCR:LA-cp rats showed less proliferation than the lean controls and there was significantly less hydroxyproline content in the obese JCR:LA-cp wounds and in the supernatants of the fibroblasts explanted from obese rats. Although SMCs and fibroblasts both differentiate from mesenchymal cells, they may exhibit different behaviors in the JCR rat due to the other cells and factors they are exposed to.

It has been demonstrated that human fibroblasts synthesize leptin and express all isoforms of the leptin receptor including the long form (OB-Rb) which is capable of leptin signaling (4). This same study demonstrated that incubation of fibroblasts with

physiological levels of insulin (1nmol/L) increased leptin secretion in fibroblast supernatants to 152% of basal levels. We have suggested that one explanation for a lack of significant differences observed in our fibroblast *in vitro* assays may be that a factor or factors may be absent which would greatly enhance the behavior of the fibroblasts from the lean rats while having a minimal effect on the fibroblasts from obese rats. One such factor may be insulin. Insulin is known for its growth-promoting properties and it would be interesting to show whether insulin treatment causes more pronounced differences in the behavior of fibroblasts.

Experiments would include fibroblast proliferation over a period of 16 days with various concentrations of insulin (0.1-10 nmol/L) and in the absence of insulin as a control. Leptin concentrations from fibroblast supernatants would also be measured over a period of 48 hours by radioimmunoassay (4). To determine the effect leptin on collagen synthesis and cytokine release, we would treat fibroblasts from lean rats with insulin to induce leptin production and measure baseline behavior. We would then treat fibroblasts with insulin and antibodies to leptin to see if inhibiting the autocrine effects of leptin causes alterations in collagen and cytokine production by fibroblasts from lean rats. TGF-β1 would be measured in supernatants before insulin treatment, at 24 hours and at 48 hours post-treatment. Hydroxyproline would be measured in supernatants at 72 hours post-treatment. All experiments would be carried out in low serum (2% dialyzed FBS).

D. Gender and Adipokine Levels

An interesting characteristic of the JCR:LA-cp rat is the fact that the males succumb to a more severe form of atherosclerosis than do the females (22). It is also of interest that the plasma levels of some adipokines such as adiponectin (23) and leptin (24) are gender dependent as well as dependent on body mass index (BMI). In one study, men and women with BMI>30kg/m^2 were compared. Although the women had a two-fold higher percent body fat, their plasma adiponectin levels were 65% higher (23). Interestingly, adiponectin is thought to be protective against atherosclerosis due to anti-inflammatory effects on endothelial cells and macrophages (25, 26). Female mice and humans are also hyperleptinaemic compared to males and have a higher incidence of autoimmune disease due to the increase in Th1 and suppression of Th2 cytokine production by leptin; this may be due to hormones or sex-linked genetic factors (24).

Estrogen has been found to play an important role in wound healing. Post-menopausal women experience reduced skin thickness due to decreased collagen content, this is reversed with topical estrogen treatment (27). Estrogen normalizes collagen content and structure and also lowers MMP-2 and MMP-9 in ovariectomized (OVX) rats (28). By normalizing collagen content, estrogen has also been shown to increase wound contraction (29). Chronic wound healing is often characterized by a delayed and prolonged inflammatory phase. Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine involved in hormonally-regulated inflammation. MIF is produced by T cells, monocytes, endothelial cells, keratinocytes, and anterior pituitary cells and it is involved in lymphocyte activation, nitric oxide synthesis, and TNF-α synthesis (30). Interestingly, MIF synthesis is also induced by TNF-α and IFN-γ (31).

Ashcroft *et al.* (32) were the first to report a link between estrogen and MIF in wound healing. In OVX mice, the absence of estrogen results in upregulation of MIF. In *MIF* null mice, the absence of estrogen had no effect on wound healing. And finally, the absence of estrogen in wild-type mice treated with anti-MIF antibodies showed a reversal in healing delay and inflammatory response.

Female JCR:LA-cp rats have not been used to study wound healing. We propose to measure serum levels of TNF-α, leptin, and adiponectin from both lean and obese male and female rats. We would also collect tissue samples for immunohistochemistry and adipose tissue samples rats to analyze adipokine levels using northern analysis and/or 2-dimensional electrophoresis. It would be of particular interest to measure the estrogen levels of lean and obese female rats to determine if there are correlations between estrogen, weight, insulin level, and adipokine levels. Additional studies with ovariectomized rats would have to be performed to further understand to role of estrogen in this model. We would expect that the adipokines of the obese female JCR rats to be closer to control levels but not in the normal range, and for the healing of the female rats to be less impaired than the obese male JCR rats.

E. Identification of the Adiponectin Receptor(s) on Cells Involved in Wound Healing

The adiponectin receptors AdipoR1 and AdipoR2 were recently cloned (33). Human and mouse AdipoR1 are 96.8% identical, while human and mouse AdipoR2 share 95% identity. Mouse AdipoR1 and AdipoR2 proteins share 66.7% identity and are

thought to have different binding affinities for either globular or full-length adiponectin (34). AdipoR1 has a high affinity for globular adiponectin but low affinity for full-length adiponectin while AdipoR2 has an intermediate affinity for both globular and full length adiponectin. Northern blotting determined that AdipoR1 expression was ubiquitous in the mouse tissues analyzed (brain, heart, kidney, liver, lung, skeletal muscle, spleen and testis), but was highest in skeletal muscle while AdipoR2 expression was highest in liver (33). The expression of both AdipoR1 and AdipoR2 has recently been shown in both human and rat pancreatic β cells (35). PCR fragments of rat and human AdipoR1 and AdipoR2 genes were DNA sequenced and were found share 92-99% identity.

The function of adiponectin signaling in different tissues appears to vary. In myocytes, adiponectin mediates fatty-acid oxidation and glucose uptake (33). In pancreatic β cells, adiponectin did not induce FFA oxidation, but FFAs did upregulate adiponectin receptors and globular adiponectin increased the expression of lipoprotein lipase (35). Lipoprotein lipase is involved in delivering FFAs to tissues and it was suggested that adiponectin may aid in the delivery of nutrients to β-cells.

Adiponectin has been shown to inhibit TNF-α-induced expression of VCAM-1, E-selectin, and ICAM-1 and to inhibit monocyte adhesion to human aortic endothelial cells (25). Since adiponectin decreases inflammatory cell adhesion to vessel walls, it is possible that adiponectin plays an important role in controlling inflammation during wound healing. The prolonged presence of inflammatory cells in chronic wounds (10) may be a result of decreased adiponectin signaling. Adiponectin is derived from

adipocytes only and the localization of adiponectin receptors in the skin has not been determined.

We propose to identify the cell types involved in wound healing which express adiponectin receptors. We would use RT-PCR to measure AdipoR1 and AdipoR2 expression on fibroblasts, keratinocytes, and endothelial cells in lean JCR:LA-cp rats. Obese JCR:LA-cp rat cells would also be assessed to see whether the expression of the adiponectin receptors are altered in obesity. The next important question that must be addressed is- what function does adiponectin signaling have on the behaviour of these cells? We can begin to answer this question by culturing cells with and without adiponectin and assess cell behaviour.

III. Final conclusion

Adipose tissue is in close proximity to the dermis. Recent evidence shows that there are several cytokines or adipokines produced by the adipose tissue that have not yet been characterized insofar as their involvement in wound healing. This opens the door to a new line of cutting-edge wound healing research. With the increasing percentage of our society becoming obese, these healing issues must be addressed.

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